TREATMENT OF PULMONARY DISORDERS USING TNFα INHIBITORS

RELATED APPLICATIONS

This application claims priority to prior filed U.S. Provisional Application Serial No. 60/397,275, filed July 19, 2002. This application also claims priority to prior filed to U.S. Provisional Application Serial No. 60/411,081, filed September 16, 2002, and prior-filed U.S. Provisional Application Serial No. 60/417490, filed October 10, 2002. This application also claims priority to prior filed to U.S. Provisional Application Serial No. 60/455777, filed March 18, 2003. In addition, this application is related to U.S. Patent Nos. 6,090,382, 6,258,562, and 6,509,015. This application is also related to U.S. Patent Application Serial No. 09/801,185, filed March 7, 2001; U.S. Patent Application Serial No. 10/302,356, filed November 22, 2002; U.S. Patent Application Serial No. 10/163657, filed June 2, 2002; and U.S. Patent Application Serial No. 10/133715, filed April 26, 2002.

This application is related to U.S. utility applications (Attorney Docket No. BPI-187) entitled "Treatment of TNFα-Related Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-188) entitled "Treatment of Spondyloarthropathies Using TNFα Inhibitors," (Attorney Docket No. BPI-189) entitled "Treatment of Pulmonary Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-190) entitled "Treatment of Coronary Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-191) entitled "Treatment of Metabolic Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-192) entitled "Treatment of Anemia Using TNFα Inhibitors," (Attorney Docket No. BPI-193) entitled "Treatment of Pain Using TNFα Inhibitors," (Attorney Docket No. BPI-194) entitled "Treatment of Hepatic Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-195) entitled "Treatment of Skin and Nail Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-196) entitled "Treatment of Vasculitides Using TNFα Inhibitors," (Attorney Docket No. BPI-197) entitled "Treatment of TNFα-Related Disorders Using TNFα Inhibitors," and PCT application (Attorney Docket No. BPI-187PC) entitled "Treatment of TNFα-Related Disorders," all of which are filed on

even date herewith. The entire contents of each of these patents and patent applications are hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

Cytokines, such as interleukin-1 (IL-1)and tumor necrosis factor (TNF) are molecules produced by a variety of cells, such as monocytes and macrophages, which have been identified as mediators of inflammatory processes. Cytokines, including TNF, regulate the intensity and duration of the inflammatory response which occurs as the result of an injury, disease or infection. Cytokines have also been implicated in the pathophysiology of lung disease (Bingisser, et al. (1996) *Am J Respir Cell Mol Biol.* 15:64; Silver (1995) *Int Rev Immunol.* 12:281; Agostini *et al.* (1996) *Am J Respir Crit Care Med.* 153:1359). For example, TNFα (also referred to as TNF) and TGFβ expression was in children with interstitial lung disease (Chadelat et al. (1998) *Eur Respir J.* 11:1329). In another study of patients with squamous cell carcinoma, there was found to be a correlation between TNFα concentration in the bronchoalveolar lavage fluid and the patient's stage of malignancy (Chyczewska et al. (1997) *Rocz Akad Med Bialymst.* 42 Suppl 1:123).

High-dose corticosteroids are universally accepted as the approach to treatment of idiopathic interstitial lung diseases and chronic obstructive airways disorders, however, treatment in many cases of these disorders is not curative and toxicity from typical therapy (*e.g.,* high-dose corticosteroids) is universal. This in turn leads to permanent sequelae in many patients.

SUMMARY OF THE INVENTION

The present invention provides a therapeutic agent capable of treating idiopathic interstitial lung diseases and chronic obstructive airways disorders without the side-effects associated with steroid use. The present invention includes methods for treating idiopathic interstitial lung diseases and chronic obstructive airways disorders in a safe and effective manner where TNFα activity is detrimental. People suffering from idiopathic interstitial lung diseases and chronic obstructive airways disorders have

elevated levels of tumor necrosis factor α (TNFα) (Soler et al. (1999) *Eur Respir J.* 14(5):1015; Ketaings et al. (1996) *Am J Respir Crit Care Med.* 153(2):530).

One aspect of the invention describes a method of treating idiopathic interstitial lung disease or a chronic obstructive airway disorder in a subject comprising administering to the subject a therapeutically effective amount of a neutralizing, high affinity TNFα antibody, such that said disorder is treated. In one embodiment, the antibody is an isolated human antibody, or an antigen-binding portion thereof, that dissociates from human TNFα with a K_d of 1 x 10^{-8} M or less and a K_{off} rate constant of 1 x 10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1 x 10^{-7} M or less. In another embodiment, the antibody is an isolated human antibody, or an antigen-binding portion thereof dissociates from human TNFα with a K_{off} rate constant of 1 x 10^{-3} s^{-1} or less, as determined by surface plasmon resonance; has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9; and has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12. In another embodiment of the invention, the antibody is an isolated human antibody, or an antigen-binding portion thereof, with a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO:1 and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2. In still another embodiment, the antibody is D2E7, also referred to as HUMIRA® (adalimumab). In one embodiment of the invention, the idiopathic interstitial lung disease is idiopathic pulmonary fibrosis. In another embodiment of the invention, the chronic obstructive airway disorder is asthma or chronic obstructive pulmonary disease (COPD).

Another aspect of the invention includes a method of treating a subject suffering from an idiopathic interstitial lung disease comprising administering a therapeutically effective amount of a TNFα antibody, or an antigen-binding fragment thereof, to the

subject, wherein the antibody dissociates from human TNFα with a K_d of 1 x 10^{-8} M or less and a K_{off} rate constant of 1 x 10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1 x 10^{-7} M or less, such that the idiopathic interstitial lung disease is treated.

Yet another aspect of the invention describes a method of treating a subject suffering from an idiopathic interstitial lung disease comprising administering a therapeutically effective amount a TNFα antibody, or an antigen-binding fragment thereof, wherein the antibody dissociates from human TNFα with a K_{off} rate constant of 1 x 10^{-3} s^{-1} or less, as determined by surface plasmon resonance; has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9; and has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12, such that the idiopathic interstitial lung disease is treated.

Another aspect of the invention features a method of treating a subject suffering from an idiopathic interstitial lung disease comprising administering a therapeutically effective amount a TNFα antibody, or an antigen-binding fragment thereof, with a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2, such that the idiopathic interstitial lung disease is treated.

One aspect of the invention includes a method of treating a subject suffering from a chronic obstructive airway disorder comprising administering a therapeutically effective amount of a TNFα antibody, or an antigen-binding fragment thereof, to the subject, wherein the antibody dissociates from human TNFα with a K_d of 1 x 10^{-8} M or less and a K_{off} rate constant of 1 x 10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1 x 10^{-7} M or less, such that the chronic obstructive airway disorder is treated.

Another aspect of the invention includes a method of treating a subject suffering from a chronic obstructive airway disorder comprising administering a therapeutically effective amount a TNFα antibody, or an antigen-binding fragment thereof, wherein the antibody dissociates from human TNFα with a K_{off} rate constant of 1 x 10^{-3} s^{-1} or less, as determined by surface plasmon resonance; has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9; and has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12 such that the chronic obstructive airway disorder is treated.

Yet another aspect of the invention includes a method of treating a subject suffering from a chronic obstructive airway disorder comprising administering a therapeutically effective amount a TNFα antibody, or an antigen-binding fragment thereof, with a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2 such that the chronic obstructive airway disorder is treated.

In one embodiment of the invention, the antibody, or antigen-binding fragment thereof, is D2E7. In another embodiment of the invention, the idiopathic interstitial lung disease is idiopathic pulmonary fibrosis. In still another embodiment of the invention, the chronic obstructive airway disorder is asthma or COPD.

One aspect of the invention includes a method of treating a subject suffering from a pulmonary disorder selected from the group consisting of asthma, chronic obstructive pulmonary disease, and idiopathic pulmonary fibrosis comprising administering a therapeutically effective amount of a TNFα antibody, or an antigen-binding fragment thereof, to the subject, wherein the antibody dissociates from human TNFα with a K_d of 1 x 10^{-8} M or less and a K_{off} rate constant of 1 x 10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1 x 10^{-7} M or less, such that said pulmonary disorder is treated.

Another aspect of the invention includes a method of treating a subject suffering from a pulmonary disorder selected from the group consisting of asthma, COPD, and idiopathic pulmonary fibrosis comprising administering a therapeutically effective amount a TNFα antibody, or an antigen-binding fragment thereof, wherein the antibody dissociates from human TNFα with a K_{off} rate constant of 1×10^{-3} s^{-1} or less, as determined by surface plasmon resonance; has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9; and has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.

In one aspect of the invention, a method of treating a subject suffering from a pulmonary disorder selected from the group consisting of asthma, COPD, and idiopathic pulmonary fibrosis is described comprising administering a therapeutically effective amount a TNFα antibody, or an antigen-binding fragment thereof, with a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2.

In one embodiment of the invention, the TNFα antibody, or antigen binding fragment thereof, is D2E7.

In another embodiment of the invention, the TNFα antibody is administered with at least one additional therapeutic agent.

Another aspect of the invention includes a method for inhibiting human TNFα activity in a human subject suffering from an idiopathic interstitial lung disease comprising administering a therapeutically effective amount of a TNFα antibody, or an antigen-binding fragment thereof, to the subject, wherein the antibody dissociates from human TNFα with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1×10^{-7} M or less. In one embodiment, the idiopathic interstitial lung disease is idiopathic pulmonary fibrosis.

In yet another aspect of the invention, a method for inhibiting human TNFα activity in a human subject suffering from a chronic obstructive airway disorder is described comprising administering a therapeutically effective amount of a TNFα antibody, or an antigen-binding fragment thereof, to the subject, wherein the antibody dissociates from human TNFα with a K_d of 1 x 10^{-8} M or less and a K_{off} rate constant of 1 x 10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1 x 10^{-7} M or less. In one embodiment, the chronic obstructive airway disorder is asthma or COPD. In another embodiment, the TNFα antibody, or antigen-binding fragment thereof, is D2E7.

An additional aspect of the invention includes a method for inhibiting human TNFα activity in a human subject suffering from a pulmonary disorder selected from the group consisting of asthma, COPD, and idiopathic pulmonary fibrosis, comprising administering a therapeutically effective amount of a TNFα antibody, or an antigen-binding fragment thereof, to the subject, wherein the antibody dissociates from human TNFα with a K_d of 1 x 10^{-8} M or less and a K_{off} rate constant of 1 x 10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1 x 10^{-7} M or less. In one embodiment, the antibody, or antigen binding fragment thereof, is D2E7.

Still another aspect of the invention describes a method of treating a subject suffering from an idiopathic interstitial lung disease comprising administering a therapeutically effective amount of D2E7, or an antigen-binding fragment thereof, to the subject, such that the disease is treated. In one embodiment, the idiopathic interstitial lung disease is idiopathic pulmonary fibrosis.

The invention also features a method of treating a subject suffering from a chronic obstructive airway disorder comprising administering a therapeutically effective amount of D2E7, or an antigen-binding fragment thereof, to the subject, such that the disorder is treated. In one embodiment, the chronic obstructive airway disorder is asthma or COPD.

Another aspect of the invention includes a method of treating a subject suffering from a pulmonary disorder selected from the group consisting of asthma, idiopathic

pulmonary fibrosis, and COPD comprising administering a therapeutically effective amount of D2E7, or an antigen-binding fragment thereof, to the subject, such that the disorder is treated.

Still another aspect of the invention includes a method of treating a subject suffering from asthma, idiopathic pulmonary fibrosis, and COPD comprising administering a therapeutically effective amount of D2E7, or an antigen-binding fragment thereof, and at least one additional therapeutic agent to the subject, such that the disorder is treated.

The invention also includes a kit comprising a pharmaceutical composition comprising a TNFα antibody, or an antigen binding portion thereof, and a pharmaceutically acceptable carrier; and instructions for administering to a subject the TNFα antibody pharmaceutical composition for treating a subject who is suffering from asthma, COPD, or idiopathic pulmonary fibrosis. In one embodiment, the TNFα antibody, or an antigen binding portion thereof, is D2E7.

DETAILED DESCRIPTION OF THE INVENTION

This invention pertains to methods of treating idiopathic interstitial lung diseases and chronic obstructive airway disorders in which TNFα activity, e.g., human TNFα activity, is detrimental. The methods include administering to the subject an effective amount of a TNFα inhibitor, such that the idiopathic interstitial lung disease or chronic obstructive airway disorder is treated. The invention also pertains to methods wherein the TNFα inhibitor is administered in combination with another therapeutic agent to treat an idiopathic interstitial lung disease or a chronic obstructive airway disorder. Various aspects of the invention relate to treatment with antibodies and antibody fragments, and pharmaceutical compositions comprising a TNFα inhibitor, and a pharmaceutically acceptable carrier for the treatment of an interstitial lung disease or a chronic obstructive airway disorder.

Definitions

In order that the present invention may be more readily understood, certain terms are first defined.

The term "human TNFα" (abbreviated herein as hTNFα, or simply hTNF), as used herein, is intended to refer to a human cytokine that exists as a 17 kD secreted form and a 26 kD membrane associated form, the biologically active form of which is composed of a trimer of noncovalently bound 17 kD molecules. The structure of hTNFα is described further in, for example, Pennica, D., *et al.* (1984) *Nature* 312:724-729; Davis, J.M., *et al.* (1987) *Biochemistry* 26:1322-1326; and Jones, E.Y., *et al.* (1989) *Nature* 338:225-228. The term human TNFα is intended to include recombinant human TNFα (rhTNFα), which can be prepared by standard recombinant expression methods or purchased commercially (R & D Systems, Catalog No. 210-TA, Minneapolis, MN). TNFα is also referred to as TNF.

The term "TNFα inhibitor" includes agents which inhibit TNFα. Examples of TNFα inhibitors include etanercept (Enbrel®, Amgen), infliximab (Remicade®, Johnson and Johnson), human anti-TNF monoclonal antibody (D2E7/HUMIRA®, Abbott Laboratories), CDP 571 (Celltech), and CDP 870 (Celltech) and other compounds which inhibit TNFα activity, such that when administered to a subject suffering from or at risk of suffering from a disorder in which TNFα activity is detrimental, the disorder is treated. In one embodiment, a TNFα inhibitor is a compound, excluding etanercept and infliximab, which inhibits TNFα activity. In another embodiment, the TNFα inhibitors of the invention are used to treat a TNFα-related disorder, as described in more detail in section II. In one embodiment, the TNFα inhibitor, excluding etanercept and infliximab, is used to treat a TNFα-related disorder. The term also includes each of the anti-TNFα human antibodies and antibody portions described herein as well as those described in U.S. Patent Nos. 6,090,382; 6,258,562; 6,509,015, and in U.S. Patent Application Serial Nos. 09/801185 and 10/302356.

The term "antibody", as used herein, is intended to refer to immunoglobulin molecules comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein

as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The antibodies of the invention are described in further detail in U.S. Patent Nos. 6,090,382; 6,258,562; and 6,509,015, and in U.S. Patent Application Serial Nos. 09/801185 and 10/302356, each of which is incorporated herein by reference in its entirety.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, hTNFα). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883) . Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short

to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see *e.g.*, Holliger, P., *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Poljak, R.J., *et al.* (1994) *Structure* 2:1121-1123). The antibody portions of the invention are described in further detail in U.S. Patent Nos. 6,090,382, 6,258,562, 6,509,015, and in U.S. Patent Application Serial Nos. 09/801185 and 10/302356, each of which is incorporated herein by reference in its entirety.

Binding fragments are produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins. Binding fragments include Fab, Fab', $F(ab')_2$, Fabc, Fv, single chains, and single-chain antibodies. Other than "bispecific" or "bifunctional" immunoglobulins or antibodies, an immunoglobulin or antibody is understood to have each of its binding sites identical. A "bispecific" or "bifunctional antibody" is an artificial hybrid antibody having two different heavy/light chain pairs and two different binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of Fab' fragments. See, *e.g.*, Songsivilai & Lachmann, *Clin. Exp. Immunol.* 79:315-321 (1990); Kostelny *et al.*, *J. Immunol.* 148, 1547-1553 (1992).

A "conservative amino acid substitution", as used herein, is one in which one amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine).

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), for example

in the CDRs and in particular CDR3. However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The term "recombinant human antibody", as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell (described further below), antibodies isolated from a recombinant, combinatorial human antibody library (described further below), antibodies isolated from an animal (*e.g.*, a mouse) that is transgenic for human immunoglobulin genes (see *e.g.*, Taylor, L.D., *et al.* (1992) *Nucl. Acids Res.* 20:6287-6295) or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies are subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

An "isolated antibody", as used herein, is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds hTNFα is substantially free of antibodies that specifically bind antigens other than hTNFα). An isolated antibody that specifically binds hTNFα may, however, have cross-reactivity to other antigens, such as hTNFα molecules from other species (discussed in further detail below). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

A "neutralizing antibody", as used herein (or an "antibody that neutralized hTNFα activity"), is intended to refer to an antibody whose binding to hTNFα results in inhibition of the biological activity of hTNFα. This inhibition of the biological activity of hTNFα can be assessed by measuring one or more indicators of hTNFα biological

activity, such as hTNFα-induced cytotoxicity (either *in vitro* or *in vivo*), hTNFα-induced cellular activation and hTNFα binding to hTNFα receptors. These indicators of hTNFα biological activity can be assessed by one or more of several standard *in vitro* or *in vivo* assays known in the art (see U.S. Patent No. 6,090,382). Preferably, the ability of an antibody to neutralize hTNFα activity is assessed by inhibition of hTNFα-induced cytotoxicity of L929 cells. As an additional or alternative parameter of hTNFα activity, the ability of an antibody to inhibit hTNFα-induced expression of ELAM-1 on HUVEC, as a measure of hTNFα-induced cellular activation, can be assessed.

The term "surface plasmon resonance", as used herein, refers to an optical phenomenon that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix, for example using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden and Piscataway, NJ). For further descriptions, see Example 1 and Jönsson, U., *et al.* (1993) *Ann. Biol. Clin.* 51:19-26; Jönsson, U., *et al.* (1991) *Biotechniques* 11:620-627; Johnsson, B., *et al.* (1995) *J. Mol. Recognit.* 8:125-131; and Johnnson, B., *et al.* (1991) *Anal. Biochem.* 198:268-277.

The term "K_{off}", as used herein, is intended to refer to the off rate constant for dissociation of an antibody from the antibody/antigen complex.

The term "K_d", as used herein, is intended to refer to the dissociation constant of a particular antibody-antigen interaction.

The term "IC_{50}" as used herein, is intended to refer to the concentration of the inhibitor required to inhibit the biological endpoint of interest, e.g., neutralize cytotoxicity activity.

The term "nucleic acid molecule", as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "isolated nucleic acid molecule", as used herein in reference to nucleic acids encoding antibodies or antibody portions (*e.g.*, VH, VL, CDR3) that bind hTNFα, is intended to refer to a nucleic acid molecule in which the nucleotide sequences encoding the antibody or antibody portion are free of other nucleotide sequences encoding antibodies or antibody portions that bind antigens other than hTNFα, which

other sequences may naturally flank the nucleic acid in human genomic DNA. Thus, for example, an isolated nucleic acid of the invention encoding a VH region of an anti-hTNFα antibody contains no other sequences encoding other VH regions that bind antigens other than hTNFα.

The term "vector", as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The term "recombinant host cell" (or simply "host cell"), as used herein, is intended to refer to a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein.

The term "dosing", as used herein, refers to the administration of a substance (*e.g.*, an anti-TNFα antibody) to achieve a therapeutic objective (*e.g.*, the treatment of a TNFα-associated disorder).

The terms "biweekly dosing regimen", "biweekly dosing", and "biweekly administration", as used herein, refer to the time course of administering a substance (*e.g.*, an anti-TNFα antibody) to a subject to achieve a therapeutic objective (*e.g.*, the treatment of a TNFα-associated disorder). The biweekly dosing regimen is not intended to include a weekly dosing regimen. Preferably, the substance is administered every 9-19 days, more preferably, every 11-17 days, even more preferably, every 13-15 days, and most preferably, every 14 days.

The term "combination" as in the phrase "a first agent in combination with a second agent" includes co-administration of a first agent and a second agent, which for example may be dissolved or intermixed in the same pharmaceutically acceptable carrier, or administration of a first agent, followed by the second agent, or administration of the second agent, followed by the first agent. The present invention, therefore, includes methods of combination therapeutic treatment and combination pharmaceutical compositions.

The term "concomitant" as in the phrase "concomitant therapeutic treatment" includes administering an agent in the presence of a second agent. A concomitant therapeutic treatment method includes methods in which the first, second, third, or additional agents are co-administered. A concomitant therapeutic treatment method also includes methods in which the first or additional agents are administered in the presence of a second or additional agents, wherein the second or additional agents, for example, may have been previously administered. A concomitant therapeutic treatment method may be executed step-wise by different actors. For example, one actor may administer to a subject a first agent and a second actor may to administer to the subject a second agent, and the administering steps may be executed at the same time, or nearly the same time, or at distant times, so long as the first agent (and additional agents) are after administration in the presence of the second agent (and additional agents). The actor and the subject may be the same entity (*e.g.*, human).

The term "combination therapy", as used herein, refers to the administration of two or more therapeutic substances, *e.g.*, an anti-TNFα antibody and another drug, such as a DMARD or NSAID. The other drug(s) may be administered concomitant with, prior to, or following the administration of an anti-TNFα antibody.

The term "pulmonary disease" as used herein refers to any idiopathic interstitial lung disease and/or chronic obstructive airway disorder. In one embodiment of the invention, the term pulmonary disease includes any lung disease and/or chronic obstructive airway disorder excluding shock lung, chronic pulmonary inflammatory disease, pulmonary sacroidosis, pulmonary fibrosis, and silicosis.

The term " idiopathic interstitial lung disease" or "idiopathic interstitial lung disorder," as used interchangeably herein, refers to any one of several diseases of unknown etiology with similar clinical features, producing diffuse pathologic changes primarily in interalveolar interstitial tissue. Examples of idiopathic interstitial lung diseases include, but are not limited to, interstitial pulmonary fibrosis (IPF). In one embodiment, idiopathic interstitial lung diseases include any one of several diseases of unknown etiology with similar clinical features, producing diffuse pathologic changes primarily in interalveolar interstitial tissue but exclude shock lung, chronic pulmonary inflammatory disease, pulmonary sacroidosis, pulmonary fibrosis, and silicosis.

The term "chronic obstructive airway disorder" as used herein, refers to pulmonary diseases due to physiologically determined chronic airflow obstruction, regardless of etiology. Examples of chronic obstructive airway disorders include, but are not limited to, asthma and chronic obstructive pulmonary disease (COPD). In one embodiment, the term chronic obstructive airway disorder includes pulmonary diseases due to physiologically determined chronic airflow obstruction but excludes shock lung, chronic pulmonary inflammatory disease, pulmonary sacroidosis, pulmonary fibrosis, and silicosis

The term "airway obstruction" refers to an increased resistance to airflow exhibited by characteristic spirometric findings.

The term "kit" as used herein refers to a packaged product comprising components with which to administer the TNFα antibody of the invention for treatment of a TNFα-related disorder. The kit preferably comprises a box or container that holds

the components of the kit. The box or container is affixed with a label or a Food and Drug Administration approved protocol. The box or container holds components of the invention which are preferably contained within plastic, polyethylene, polypropylene, ethylene, or propylene vessels. The vessels can be capped-tubes or bottles. The kit can also include instructions for administering the TNFα antibody of the invention.

Various aspects of the invention are described in further detail herein.

I. TNFα Inhibitors of the Invention

This invention provides methods of treating idiopathic interstitial lung diseases or chronic obstructive airway disorders in which the administration of a TNFα inhibitor is beneficial. In one embodiment, these methods include administration of isolated human antibodies, or antigen-binding portions thereof, that bind to human TNFα with high affinity, a low off rate and high neutralizing capacity. Preferably, the human antibodies of the invention are recombinant, neutralizing human anti-hTNFα antibodies. The most preferred recombinant, neutralizing antibody of the invention is referred to herein as D2E7 (the amino acid sequence of the D2E7 VL region is shown in SEQ ID NO: 1; the amino acid sequence of the D2E7 VH region is shown in SEQ ID NO: 2). D2E7 is also referred to as HUMIRA® and adalimumab. The properties of D2E7 have been described in Salfeld *et al.*, U.S. patent No. 6,090,382, which is incorporated by reference herein.

In one embodiment, the treatment of the invention includes the administration of D2E7 antibodies and antibody portions, D2E7-related antibodies and antibody portions, and other human antibodies and antibody portions with equivalent properties to D2E7, such as high affinity binding to hTNFα with low dissociation kinetics and high neutralizing capacity. In one embodiment, the invention provides treatment with an isolated human antibody, or an antigen-binding portion thereof, that dissociates from human TNFα with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1×10^{-7} M or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of 5×10^{-4} s^{-1} or less, or even more preferably, with a

K_{off} of 1 x 10^{-4} s^{-1} or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1 x 10^{-8} M or less, even more preferably with an IC_{50} of 1 x 10^{-9} M or less and still more preferably with an IC_{50} of 1 x 10^{-10} M or less. In a preferred embodiment, the antibody is an isolated human recombinant antibody, or an antigen-binding portion thereof.

It is well known in the art that antibody heavy and light chain CDR3 domains play an important role in the binding specificity/affinity of an antibody for an antigen. Accordingly, in another aspect, the invention pertains to methods of treating inflammatory disorders in which the TNFα activity is detriment by administering human antibodies that have slow dissociation kinetics for association with hTNFα and that have light and heavy chain CDR3 domains that structurally are identical to or related to those of D2E7. Position 9 of the D2E7 VL CDR3 can be occupied by Ala or Thr without substantially affecting the K_{off}. Accordingly, a consensus motif for the D2E7 VL CDR3 comprises the amino acid sequence: Q-R-Y-N-R-A-P-Y-(T/A) (SEQ ID NO: 3). Additionally, position 12 of the D2E7 VH CDR3 can be occupied by Tyr or Asn, without substantially affecting the K_{off}. Accordingly, a consensus motif for the D2E7 VH CDR3 comprises the amino acid sequence: V-S-Y-L-S-T-A-S-S-L-D-(Y/N) (SEQ ID NO: 4). Moreover, as demonstrated in Example 2 of U.S. Patent No. 6,090,382, the CDR3 domain of the D2E7 heavy and light chains is amenable to substitution with a single alanine residue (at position 1, 4, 5, 7 or 8 within the VL CDR3 or at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 within the VH CDR3) without substantially affecting the K_{off}. Still further, the skilled artisan will appreciate that, given the amenability of the D2E7 VL and VH CDR3 domains to substitutions by alanine, substitution of other amino acids within the CDR3 domains may be possible while still retaining the low off rate constant of the antibody, in particular substitutions with conservative amino acids. Preferably, no more than one to five conservative amino acid substitutions are made within the D2E7 VL and/or VH CDR3 domains. More preferably, no more than one to three conservative amino acid substitutions are made within the D2E7 VL and/or VH CDR3 domains. Additionally, conservative amino acid substitutions should not be made at amino acid positions critical for binding to hTNFα. Positions 2 and 5 of the D2E7 VL

CDR3 and positions 1 and 7 of the D2E7 VH CDR3 appear to be critical for interaction with hTNFα and thus, conservative amino acid substitutions preferably are not made at these positions (although an alanine substitution at position 5 of the D2E7 VL CDR3 is acceptable, as described above) (*see* U.S. Patent No. 6,090,382).

Accordingly, in another embodiment, the invention provides methods of treating idiopathic interstitial lung diseases and chronic obstructive airway disorders by the administration of an isolated human antibody, or antigen-binding portion thereof. The antibody or antigen-binding portion thereof preferably contains the following characteristics:

a) dissociates from human TNFα with a K_{off} rate constant of $1 \times 10^{-3}\ s^{-1}$ or less, as determined by surface plasmon resonance;

b) has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9;

c) has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.

More preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of $5 \times 10^{-4}\ s^{-1}$ or less. Even more preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of $1 \times 10^{-4}\ s^{-1}$ or less.

In yet another embodiment, the invention provides methods of treating idiopathic interstitial lung diseases and chronic obstructive airway disorders by the administration of an isolated human antibody, or antigen-binding portion thereof. The antibody or antigen-binding portion thereof preferably contains a light chain variable region (LCVR) having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8, and with a heavy chain variable region (HCVR) having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single

alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11. Preferably, the LCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5 (*i.e.*, the D2E7 VL CDR2) and the HCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6 (*i.e.*, the D2E7 VH CDR2). Even more preferably, the LCVR further has CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7 (*i.e.*, the D2E7 VL CDR1) and the HCVR has a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8 (*i.e.*, the D2E7 VH CDR1). The framework regions for VL preferably are from the $V_{\kappa}I$ human germline family, more preferably from the A20 human germline Vk gene and most preferably from the D2E7 VL framework sequences shown in Figures 1A and 1B of U.S. Patent No. 6,090,382. The framework regions for VH preferably are from the V_H3 human germline family, more preferably from the DP-31 human germline VH gene and most preferably from the D2E7 VH framework sequences shown in Figures 2A and 2B U.S. Patent No. 6,090,382.

Accordingly, in another embodiment, the invention provides methods of treating idiopathic interstitial lung diseases and chronic obstructive airway disorders by the administration of an isolated human antibody, or antigen-binding portion thereof. The antibody or antigen-binding portion thereof preferably contains a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 (*i.e.*, the D2E7 VL) and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2 (*i.e.*, the D2E7 VH). In certain embodiments, the antibody comprises a heavy chain constant region, such as an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region. Preferably, the heavy chain constant region is an IgG1 heavy chain constant region or an IgG4 heavy chain constant region. Furthermore, the antibody can comprise a light chain constant region, either a kappa light chain constant region or a lambda light chain constant region. Preferably, the antibody comprises a kappa light chain constant region. Alternatively, the antibody portion can be, for example, a Fab fragment or a single chain Fv fragment.

In still other embodiments, the invention provides methods of treating idiopathic interstitial lung diseases and chronic obstructive airway disorders in which the administration of an anti-TNFα antibody is beneficial administration of an isolated human antibody, or an antigen-binding portions thereof. The antibody or antigen-

binding portion thereof preferably contains D2E7-related VL and VH CDR3 domains, for example, antibodies, or antigen-binding portions thereof, with a light chain variable region (LCVR) having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25 and SEQ ID NO: 26 or with a heavy chain variable region (HCVR) having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34 and SEQ ID NO: 35.

In another embodiment, the TNFα inhibitor of the invention is etanercept (described in WO 91/03553 and WO 09/406476), infliximab (described in U.S. Patent No. 5,656,272), CDP571 (a humanized monoclonal anti-TNF-alpha IgG4 antibody), CDP 870 (a humanized monoclonal anti-TNF-alpha antibody fragment), D2E7/HUMIRA® (a human anti-TNF mAb), soluble TNF receptor Type I, or a pegylated soluble TNF receptor Type I (PEGs TNF-R1).

The TNFα antibody of the invention can be modified. In some embodiments, the TNFα antibody or antigen binding fragments thereof, is chemically modified to provide a desired effect. For example, pegylation of antibodies and antibody fragments of the invention may be carried out by any of the pegylation reactions known in the art, as described, for example, in the following references: *Focus on Growth Factors* 3:4-10 (1992); EP 0 154 316; and EP 0 401 384 (each of which is incorporated by reference herein in its entirety). Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer). A preferred water-soluble polymer for pegylation of the antibodies and antibody fragments of the invention is polyethylene glycol (PEG). As used herein, "polyethylene glycol" is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (Cl-ClO) alkoxy- or aryloxy-polyethylene glycol.

Methods for preparing pegylated antibodies and antibody fragments of the invention will generally comprise the steps of (a) reacting the antibody or antibody fragment with polyethylene glycol, such as a reactive ester or aldehyde derivative of PEG, under conditions whereby the antibody or antibody fragment becomes attached to one or more PEG groups, and (b) obtaining the reaction products. It will be apparent to one of ordinary skill in the art to select the optimal reaction conditions or the acylation reactions based on known parameters and the desired result.

Pegylated antibodies and antibody fragments may generally be used to treat pulmonary disorders of the invention by administration of the TNFα antibodies and antibody fragments described herein. Generally the pegylated antibodies and antibody fragments have increased half-life, as compared to the nonpegylated antibodies and antibody fragments. The pegylated antibodies and antibody fragments may be employed alone, together, or in combination with other pharmaceutical compositions.

In yet another embodiment of the invention, TNFα antibodies or fragments thereof can be altered wherein the constant region of the antibody is modified to reduce at least one constant region-mediated biological effector function relative to an unmodified antibody. To modify an antibody of the invention such that it exhibits reduced binding to the Fc receptor, the immunoglobulin constant region segment of the antibody can be mutated at particular regions necessary for Fc receptor (FcR) interactions (see *e.g.*, Canfield, S.M. and S.L. Morrison (1991) *J. Exp. Med.* 173:1483-1491; and Lund, J. *et al.* (1991) *J. of Immunol.* 147:2657-2662). Reduction in FcR binding ability of the antibody may also reduce other effector functions which rely on FcR interactions, such as opsonization and phagocytosis and antigen-dependent cellular cytotoxicity.

An antibody or antibody portion of the invention can be derivatized or linked to another functional molecule (*e.g.*, another peptide or protein). Accordingly, the antibodies and antibody portions of the invention are intended to include derivatized and otherwise modified forms of the human anti-hTNFα antibodies described herein, including immunoadhesion molecules. For example, an antibody or antibody portion of the invention can be functionally linked (by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as

another antibody (*e.g.*, a bispecific antibody or a diabody), a detectable agent, a cytotoxic agent, a pharmaceutical agent, and/or a protein or peptide that can mediate associate of the antibody or antibody portion with another molecule (such as a streptavidin core region or a polyhistidine tag).

One type of derivatized antibody is produced by crosslinking two or more antibodies (of the same type or of different types, *e.g.*, to create bispecific antibodies). Suitable crosslinkers include those that are heterobifunctional, having two distinctly reactive groups separated by an appropriate spacer (*e.g.*, m-maleimidobenzoyl-N-hydroxysuccinimide ester) or homobifunctional (*e.g.*, disuccinimidyl suberate). Such linkers are available from Pierce Chemical Company, Rockford, IL.

Useful detectable agents with which an antibody or antibody portion of the invention may be derivatized include fluorescent compounds. Exemplary fluorescent detectable agents include fluorescein, fluorescein isothiocyanate, rhodamine, 5-dimethylamine-1-napthalenesulfonyl chloride, phycoerythrin and the like. An antibody may also be derivatized with detectable enzymes, such as alkaline phosphatase, horseradish peroxidase, glucose oxidase and the like. When an antibody is derivatized with a detectable enzyme, it is detected by adding additional reagents that the enzyme uses to produce a detectable reaction product. For example, when the detectable agent horseradish peroxidase is present, the addition of hydrogen peroxide and diaminobenzidine leads to a colored reaction product, which is detectable. An antibody may also be derivatized with biotin, and detected through indirect measurement of avidin or streptavidin binding.

An antibody, or antibody portion, of the invention can be prepared by recombinant expression of immunoglobulin light and heavy chain genes in a host cell. To express an antibody recombinantly, a host cell is transfected with one or more recombinant expression vectors carrying DNA fragments encoding the immunoglobulin light and heavy chains of the antibody such that the light and heavy chains are expressed in the host cell and, preferably, secreted into the medium in which the host cells are cultured, from which medium the antibodies can be recovered. Standard recombinant DNA methodologies are used to obtain antibody heavy and light chain genes, incorporate these genes into recombinant expression vectors and introduce the vectors

into host cells, such as those described in Sambrook, Fritsch and Maniatis (eds), *Molecular Cloning; A Laboratory Manual, Second Edition*, Cold Spring Harbor, N.Y., (1989), Ausubel, F.M. *et al.* (eds.) *Current Protocols in Molecular Biology*, Greene Publishing Associates, (1989) and in U.S. Patent No. 4,816,397 by Boss *et al.*

To express D2E7 or a D2E7-related antibody, DNA fragments encoding the light and heavy chain variable regions are first obtained. These DNAs can be obtained by amplification and modification of germline light and heavy chain variable sequences using the polymerase chain reaction (PCR). Germline DNA sequences for human heavy and light chain variable region genes are known in the art (see *e.g.*, the "Vbase" human germline sequence database; see also Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242; Tomlinson, I.M., *et al.* (1992) "The Repertoire of Human Germline V_H Sequences Reveals about Fifty Groups of V_H Segments with Different Hypervariable Loops" *J. Mol. Biol.* 227:776-798; and Cox, J.P.L. *et al.* (1994) "A Directory of Human Germ-line V_{78} Segments Reveals a Strong Bias in their Usage" *Eur. J. Immunol.* 24:827-836; the contents of each of which are expressly incorporated herein by reference). To obtain a DNA fragment encoding the heavy chain variable region of D2E7, or a D2E7-related antibody, a member of the V_H3 family of human germline VH genes is amplified by standard PCR. Most preferably, the DP-31 VH germline sequence is amplified. To obtain a DNA fragment encoding the light chain variable region of D2E7, or a D2E7-related antibody, a member of the $V_\kappa I$ family of human germline VL genes is amplified by standard PCR. Most preferably, the A20 VL germline sequence is amplified. PCR primers suitable for use in amplifying the DP-31 germline VH and A20 germline VL sequences can be designed based on the nucleotide sequences disclosed in the references cited *supra*, using standard methods.

Once the germline VH and VL fragments are obtained, these sequences can be mutated to encode the D2E7 or D2E7-related amino acid sequences disclosed herein. The amino acid sequences encoded by the germline VH and VL DNA sequences are first compared to the D2E7 or D2E7-related VH and VL amino acid sequences to identify amino acid residues in the D2E7 or D2E7-related sequence that differ from germline. Then, the appropriate nucleotides of the germline DNA sequences are mutated such that

the mutated germline sequence encodes the D2E7 or D2E7-related amino acid sequence, using the genetic code to determine which nucleotide changes should be made. Mutagenesis of the germline sequences is carried out by standard methods, such as PCR-mediated mutagenesis (in which the mutated nucleotides are incorporated into the PCR primers such that the PCR product contains the mutations) or site-directed mutagenesis.

Once DNA fragments encoding D2E7 or D2E7-related VH and VL segments are obtained (by amplification and mutagenesis of germline VH and VL genes, as described above), these DNA fragments can be further manipulated by standard recombinant DNA techniques, for example to convert the variable region genes to full-length antibody chain genes, to Fab fragment genes or to a scFv gene. In these manipulations, a VL- or VH-encoding DNA fragment is operatively linked to another DNA fragment encoding another protein, such as an antibody constant region or a flexible linker. The term "operatively linked", as used in this context, is intended to mean that the two DNA fragments are joined such that the amino acid sequences encoded by the two DNA fragments remain in-frame.

The isolated DNA encoding the VH region can be converted to a full-length heavy chain gene by operatively linking the VH-encoding DNA to another DNA molecule encoding heavy chain constant regions (CH1, CH2 and CH3). The sequences of human heavy chain constant region genes are known in the art (see *e.g.*, Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The heavy chain constant region can be an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region, but most preferably is an IgG1 or IgG4 constant region. For a Fab fragment heavy chain gene, the VH-encoding DNA can be operatively linked to another DNA molecule encoding only the heavy chain CH1 constant region.

The isolated DNA encoding the VL region can be converted to a full-length light chain gene (as well as a Fab light chain gene) by operatively linking the VL-encoding DNA to another DNA molecule encoding the light chain constant region, CL. The sequences of human light chain constant region genes are known in the art (see *e.g.*, Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth*

Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The light chain constant region can be a kappa or lambda constant region, but most preferably is a kappa constant region.

To create a scFv gene, the VH- and VL-encoding DNA fragments are operatively linked to another fragment encoding a flexible linker, *e.g.*, encoding the amino acid sequence (Gly_4-$Ser)_3$, such that the VH and VL sequences can be expressed as a contiguous single-chain protein, with the VL and VH regions joined by the flexible linker (see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883; McCafferty *et al.*, *Nature* (1990) 348:552-554).

To express the antibodies, or antibody portions of the invention, DNAs encoding partial or full-length light and heavy chains, obtained as described above, are inserted into expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" is intended to mean that an antibody gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy chain gene can be inserted into separate vector or, more typically, both genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by standard methods (*e.g.*, ligation of complementary restriction sites on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are present). Prior to insertion of the D2E7 or D2E7-related light or heavy chain sequences, the expression vector may already carry antibody constant region sequences. For example, one approach to converting the D2E7 or D2E7-related VH and VL sequences to full-length antibody genes is to insert them into expression vectors already encoding heavy chain constant and light chain constant regions, respectively, such that the VH segment is operatively linked to the CH segment(s) within the vector and the VL segment is operatively linked to the CL segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of

the antibody chain from a host cell. The antibody chain gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the antibody chain gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (*i.e.*, a signal peptide from a non-immunoglobulin protein).

In addition to the antibody chain genes, the recombinant expression vectors of the invention carry regulatory sequences that control the expression of the antibody chain genes in a host cell. The term "regulatory sequence" is intended to includes promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, *etc.* Preferred regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV) (such as the CMV promoter/enhancer), Simian Virus 40 (SV40) (such as the SV40 promoter/enhancer), adenovirus, (*e.g.*, the adenovirus major late promoter (AdMLP)) and polyoma. For further description of viral regulatory elements, and sequences thereof, see *e.g.*, U.S. Patent No. 5,168,062 by Stinski, U.S. Patent No. 4,510,245 by Bell *et al.* and U.S. Patent No. 4,968,615 by Schaffner *et al.*

In addition to the antibody chain genes and regulatory sequences, the recombinant expression vectors of the invention may carry additional sequences, such as sequences that regulate replication of the vector in host cells (*e.g.*, origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (see *e.g.*, U.S. Patents Nos. 4,399,216, 4,634,665 and 5,179,017, all by Axel *et al.*). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes

include the dihydrofolate reductase (DHFR) gene (for use in dhfr⁻ host cells with methotrexate selection/amplification) and the *neo* gene (for G418 selection).

For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains is transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, *e.g.*, electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells, and most preferably mammalian host cells, is the most preferred because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody. Prokaryotic expression of antibody genes has been reported to be ineffective for production of high yields of active antibody (Boss, M.A. and Wood, C. R. (1985) *Immunology Today* 6:12-13).

Preferred mammalian host cells for expressing the recombinant antibodies of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin, (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, *e.g.*, as described in R.J. Kaufman and P.A. Sharp (1982) *Mol. Biol.* 159:601-621), NS0 myeloma cells, COS cells and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Host cells can also be used to produce portions of intact antibodies, such as Fab fragments or scFv molecules. It will be understood that variations on the above procedure are within the scope of the present invention. For example, it may be desirable to transfect a host cell with DNA encoding either the light chain or the heavy chain (but not both) of an antibody of this invention. Recombinant DNA technology

may also be used to remove some or all of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to hTNFα. The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies of the invention. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody of the invention and the other heavy and light chain are specific for an antigen other than hTNFα by crosslinking an antibody of the invention to a second antibody by standard chemical crosslinking methods.

In a preferred system for recombinant expression of an antibody, or antigen-binding portion thereof, of the invention, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr-CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to CMV enhancer/AdMLP promoter regulatory elements to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are culture to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells and recover the antibody from the culture medium.

Recombinant human antibodies of the invention in addition to D2E7 or an antigen binding portion thereof, or D2E7-related antibodies disclosed herein can be isolated by screening of a recombinant combinatorial antibody library, preferably a scFv phage display library, prepared using human VL and VH cDNAs prepared from mRNA derived from human lymphocytes. Methodologies for preparing and screening such libraries are known in the art. In addition to commercially available kits for generating phage display libraries (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene *SurfZAP*™ phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating and screening antibody display libraries can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* PCT Publication No. WO 92/18619; Dower *et al.* PCT

Publication No. WO 91/17271; Winter *et al.* PCT Publication No. WO 92/20791; Markland *et al.* PCT Publication No. WO 92/15679; Breitling *et al.* PCT Publication No. WO 93/01288; McCafferty *et al.* PCT Publication No. WO 92/01047; Garrard *et al.* PCT Publication No. WO 92/09690; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum Antibod Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; McCafferty *et al.*, *Nature* (1990) 348:552-554; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J Mol Biol* 226:889-896; Clackson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *PNAS* 89:3576-3580; Garrard *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.* (1991) *Nuc Acid Res* 19:4133-4137; and Barbas *et al.* (1991) *PNAS* 88:7978-7982. Methods of isolating human antibodies with high affinity and a low off rate constant for hTNFα are described in U.S. Patent Nos. 6,090,382, 6,258,562, and 6,509,015, each of which is incorporated by reference herein.

II. Uses of TNFα Inhibitors of the Invention

In an embodiment, the invention provides a method for inhibiting TNFα activity in a subject suffering from idiopathic interstitial lung disease or a chronic obstructive airway disorder in which TNFα activity is detrimental. In one embodiment, the invention provides a method for inhibiting TNFα activity in a subject suffering from idiopathic pulmonary fibrosis (IPF), asthma, or chronic obstructive pulmonary disease (COPD). In one embodiment, the TNFα inhibitor is D2E7, also referred to as HUMIRA® (adalimumab).

TNFα has been implicated in the pathophysiology of a wide variety of pulmonary disorders, including pulmonary disorders such as idiopathic interstitial lung disease and chronic obstructive airway disorders (see *e.g.*, Piquet PF *et al.* (1989) *J Exp Med.* 170:655-63; Whyte M, *et al.* (2000) *Am J Respir Crit Care Med.* 162:755-8; Anticevich SZ, *et al.* (1995) *Eur J Pharmacol.* 284:221-5). The invention provides methods for TNFα activity in a subject suffering from such a pulmonary disorder, which method comprises administering to the subject an antibody, antibody portion, or other TNFα inhibitor such that TNFα activity in the subject suffering from idiopathic interstitial lung disease or a chronic obstructive airway disorder is inhibited. Preferably, the TNFα is

human TNFα and the subject is a human subject. Alternatively, the subject can be a mammal expressing a TNFα with which an antibody of the invention cross-reacts. Still further the subject can be a mammal into which has been introduced hTNFα (*e.g.*, by administration of hTNFα or by expression of an hTNFα transgene). An antibody of the invention can be administered to a human subject for therapeutic purposes (discussed further below). Moreover, an antibody of the invention can be administered to a non-human mammal expressing a TNFα with which the antibody cross-reacts (*e.g.*, a primate, pig or mouse) for veterinary purposes or as an animal model of human disease. Regarding the latter, such animal models may be useful for evaluating the therapeutic efficacy of antibodies of the invention (*e.g.*, testing of dosages and time courses of administration). Examples of animal models for evaluating the efficacy of a TNFα antibody for the treatment of idiopathic interstitial lung disease and chronic obstructive airway disorders include ovalbumin (OVA) induced allergic asthma mice and cigarette smoke induced chronic obstructive pulmonary disease mice (see Hessel, EM., *et al.* (1995) *Eur J Pharmacol.* 293:401; Keast D, *et al.* (1981) *J. Pathol.* 135:249)

As used herein, the term "idiopathic interstitial lung disease in which TNFα activity is detrimental" is intended to include pulmonary diseases and other disorders in which the presence of TNFα in a subject suffering from the disorder has been shown to be or is suspected of being either responsible for the pathophysiology of the disorder or a factor that contributes to a worsening of the disorder, *e.g.*, idiopathic pulmonary fibrosis. The term "chronic obstructive airway disorder in which TNFα activity is detrimental" is intended to include pulmonary diseases and other disorders in which the presence of TNFα in a subject suffering from the disorder has been shown to be or is suspected of being either responsible for the pathophysiology of the disorder or a factor that contributes to a worsening of the disorder, *e.g.*, asthma and chronic obstructive pulmonary disease (COPD). Accordingly, an idopathic interstitial lung disease and a chronic obstructive airway disorder in which TNFα activity is detrimental are disorders in which inhibition of TNFα activity is expected to alleviate the symptoms and/or progression of the disorder. Such disorders may be evidenced, for example, by an increase in the concentration of TNFα in a biological fluid of a subject suffering from

the disorder (*e.g.*, an increase in the concentration of TNFα in serum, plasma, synovial fluid, *etc.* of the subject), which can be detected, for example, using an anti-TNFα antibody as described above. The use of the antibodies, antibody portions, and other TNFα inhibitors of the invention in the treatment of specific inflammatory disorders including idiopathic interstitial lung diseases or chronic obstructive airway disorders is discussed further below. In certain embodiments, the antibody, antibody portion, or other TNFα inhibitor of the invention is administered to the subject in combination with another therapeutic agent, as described below in Section III. In one embodiment, the TNFα antibody of the invention is administered to the subject in combination with another therapeutic agent for the treatment of idiopathic pulmonary fibrosis, asthma, or chronic obstructive pulmonary disease.

In one embodiment, the invention features a method for treating a pulmonary disorder in which TNFα activity is detrimental, comprising administering to a subject an effective amount of a TNFα inhibitor, such that said pulmonary disorder is treated, wherein said pulmonary disorder is idiopathic interstitial lung disease or chronic obstructive airway disorder. Examples of idiopathic interstitial lung diseases and chronic obstructive airway disorders in which TNFα activity is detrimental are discussed further below.

A. *Idiopathic interstitial lung disease*

In one embodiment, the TNFα antibody of the invention is used to treat subjects who have an idiopathic interstitial lung disease. Idiopathic interstitial lung diseases affect the lungs in three ways: first, the lung tissue is damaged in some known or unknown way; second, the walls of the air sacs in the lung become inflamed; and finally, scarring (or fibrosis) begins in the interstitium (or tissue between the air sacs), and the lung becomes stiff. Examples of idiopathic interstitial lung diseases are described below.

i. *Idiopathic pulmonary fibrosis (IPF)*

Tumor necrosis factor has been implicated in the pathophysiology of idiopathic pulmonary fibrosis (IPF) (see Piquet PF, *et al.* (1989) *J Exp Med*. 170:655-63; Whyte

M, *et al.* (2000) *Am J Respir Crit Care Med* 162:755-8; Corbett EL, *et al.* (2002) *Am J Respir Crit Care Med.* 165:690-3). For example, it has been found that IPF patients have increased levels of TNF expressiojn in macrophages and in type II epithelial cells (Piquet *et al.* (1993) *Am J Pathol* 143:651; Nash *et al.* (1993) *Histopathology* 22:343; Zhang *et al.* (1993) *J Immunol* 150:4188). Certain genetic polymorphisms are also associated with increased TNF expression, and are implicated in playing a role in IPF and silicosis (Whyte *et al.*, *supra*; Corbett EL, *et al., supra*).

The term "idiopathic pulmonary fibrosis" or "IPF" refers to a group of disorders characterized by inflammation and eventually scarring of the deep lung tissues, leading to shortness of breath. The scarring of the alveoli (air sacs) and their supporting structures (the interstitium) in IPF eventually leads to a loss of the functional alveolar units and a reduction of the transfer of oxygen from air to blood. IPF is also referred to as diffuse parenchymal lung disease; alveolitis; cryptogenic fibrosing alveolitis (CFA); idiopathic pulmonary pneumonitis (IPP); and usual interstitial pneumonitis (UIP). IPF is often used synonymously with UIP ("IPF/UIP") because UIP is the most common cellular pattern seen in the pathologic diagnosis of IPF.

Patients with IPF often exhibit certain symptoms, including a dry cough, chest pain, and/or shortness of breath. Commonly used drugs for the treatment of IPF are prednisone and cytoxan, although only a fraction of patients improve with continued use of these drugs (American Thoracic Society (2000) *Am. J. Respir. Crit. Care Med.* 161:646). Oxygen administration and transplantation of the lung are other choices for treatment. In one embodiment, the TNFα antibody of the invention is administered to the subject in combination with another therapeutic agent, for example oxygen, for the treatment of idiopathic pulmonary fibrosis,.

B. *Chronic obstructive airway disorder*

In one embodiment, the TNFα antibody of the invention is used to treat a subject who has a chronic obstructive airflow disorder. In these diseases, airflow obstruction may be chronic and persistent or episodic and recurrent. Airflow obstruction is usually determined by forced expiratory spirometry, which is the recording of exhaled volume against time during a maximal expiration. In a subject who does not have an obstructed

airflow, a full forced expiration usually takes between 3 and 4 seconds. In a patient with chronic obstructive airflow disorder, wherein airflow is obstructed, it usually takes up to 15 to 20 seconds and may be limited by breath-holding time. The normal forced expiratory volume in the first second of expiration (FEV_1) is easily measured and accurately predicted on the basis of age, sex, and height. The ratio of FEV_1 to forced vital capacity (FEV_1/FVC) normally exceeds 0.75. Recording airflow against volume during forced expiration and a subsequent forced inspiration--the flow-volume loop--is also useful, mainly for distinguishing upper from lower airway narrowing. Examples of chronic obstructive airway disorders are described below.

i. *Asthma*

Tumor necrosis factor has been implicated in the pathophysiology of asthma, (Anticevich SZ, *et al.* (1995) *Eur J Pharmacol.* 284:221-5; Thomas PS, *et al.* 1995. *Am J Respir Crit Care Med.* 152:76-80; Thomas PS, Heywood G. (2002) *Thorax.* 57:774-8). For example, acute asthma attacks have been found to be associated with pulmonary neutrophilia and elevated BAL TNF levels (Ordonez CL. *et al.* (2000) *Am J Respir Crit Care Med* 161:1185). It has been found that the severity of asthma symptoms correlates with endotoxin levels in house dust. In rats, anti-TNF antibodies reduced endotoxin-induced airway changes (Kips *et al.* (1992) *Am Rev Respir Dis* 145:332).

The term "asthma" as used herein, refers to a disorder in which inflammation of the airways causes airflow into and out of the lungs to be restricted. Asthma is also referred to as bronchial asthma, exercise induced asthma - bronchial, and reactive airways disease (RAD). In some instances, asthma is associated with allergies and/or is familial. Asthma includes a condition which is characterized by widespread fluctuations in the diameter or caliber of bronchial airways over short periods of time, resulting in changes in lung function. The resulting increased resistance to air flow produces symptoms in the affected subject, including breathlessness (dyspnea), chest constriction or "tightness," and wheezing.

Patients with asthma are characterized according to NIH guidelines, are described as mild intermittent, mild persistent, moderate persistent, and severe persistent (see NAEPP Expert Panel Report Guidelines for the Diagnosis and Management of Asthma-

Update on Selected Topics 2002. JACI 2002; 110: S141-S209; Guidelines for the Diagnosis and Management of Asthma. NIH Publication 97-4051, July 1997). Patients diagnosed with moderate persistent asthma are often treated with inhaled corticosteroids. Patients diagnosed with severe persistent asthma are often treated with high dose inhaled corticosteroids and p.o. corticosteroids.

ii. *Chronic obstructive pulmonary disease (COPD)*

Tumor necrosis factor has been implicated in the pathophysiology of chronic obstructive pulmonary disease, (Keatings VM. (2000) *Chest.* 118:971-5; Sakao S, *et al.*(2001) *Am J Respir Crit Care Med.* 163:420-22; Sakao S, *et al.* (2002) *Chest.* 122:416-20). The term " chronic obstructive pulmonary disease" or "COPD" as used interchangeably herein, refers to a group of lung diseases characterized by limited airflow with variable degrees of air sack enlargement and lung tissue destruction. The term COPD includes chronic bronchitis (mucous hypersecretion with goblet cell submucosal gland hyperplasia), chronic obstructive bronchitis, or emphysema (destruction of airway parenchyma), or combinations of these conditions. Emphysema and chronic bronchitis are the most common forms of chronic obstructive pulmonary disease. COPD is defined by irreversible airflow obstruction.

In COPD, chronic inflammation leads to fixed narrowing of small airways and lung parenchyma and alveolar wall destruction (emphysema). This is characterized by increased numbers of alveolar macrophages, neutrophils, and cytotoxic T lymphocytes, and the release of multiple inflammatory mediators (lipids, chemokines, cytokines, growth factors). This inflammation leads to fibrosis with a narrowing of the small airways and lung parenchymal destruction. There is also a high level of oxidative stress, which may amplify this inflammation.

It is understood that all of the above-mentioned pulmonary disorders include both the adult and juvenile forms of the disease where appropriate. It is also understood that all of the above-mentioned disorders include both chronic and acute forms of the disease. In addition, the TNFα antibody of the invention can be used to treat each of the

above-mentioned pulmonary disorders alone or in combination with one another, *e.g.*, a subject who is suffering from COPD and IPF.

III. Pharmaceutical Compositions and Pharmaceutical Administration

A. *Compositions and Administration*

The antibodies, antibody-portions, and other TNFα inhibitors of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject. Typically, the pharmaceutical composition comprises an antibody, antibody portion, or other TNFα inhibitor of the invention and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Pharmaceutically acceptable carriers may further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the antibody, antibody portion, or other TNFα inhibitor.

The compositions of this invention may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (*e.g.*, injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. The preferred form depends on the intended mode of administration and therapeutic application. Typical preferred compositions are in the form of injectable or infusible solutions, such as compositions similar to those used for passive immunization of humans with other antibodies or other TNFα inhibitors. The preferred mode of administration is parenteral (*e.g.*, intravenous, subcutaneous, intraperitoneal, intramuscular). In a preferred embodiment, the antibody or other TNFα inhibitor is administered by intravenous infusion or injection. In another preferred

embodiment, the antibody or other TNFα inhibitor is administered by intramuscular or subcutaneous injection.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the active compound (*i.e.*, antibody, antibody portion, or other TNFα inhibitor) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Supplementary active compounds can also be incorporated into the compositions. In certain embodiments, an antibody or antibody portion of the invention is coformulated with and/or coadministered with one or more additional therapeutic agents. For example, an anti-hTNFα antibody or antibody portion of the invention may be coformulated and/or coadministered with one or more DMARD or one or more NSAID or one or more additional antibodies that bind other targets (*e.g.*, antibodies that bind other cytokines or that bind cell surface molecules), one or more cytokines, soluble TNFα receptor (see *e.g.*, PCT Publication No. WO 94/06476) and/or one or more chemical agents that inhibit hTNFα production or activity (such as cyclohexane-ylidene derivatives as described in PCT Publication No. WO 93/19751) or any combination thereof. Furthermore, one or more antibodies of the invention may be used in combination with two or more of the foregoing therapeutic agents. Such combination

therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible side effects, complications or low level of response by the patient associated with the various monotherapies.

In one embodiment, the invention includes pharmaceutical compositions comprising an effective amount of a TNFα inhibitor and a pharmaceutically acceptable carrier, wherein the effective amount of the TNFα inhibitor may be effective to treat an pulmonary disease, including, for example, a idiopathic interstitial lung disease or chronic obstructive airway disorder.

The antibodies, antibody-portions, and other TNFα inhibitors of the present invention can be administered by a variety of methods known in the art, although for many therapeutic applications, the preferred route/mode of administration is intravenous injection or infusion. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. In certain embodiments, the active compound may be prepared with a carrier that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. See, *e.g.*, *Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

The TNFα antibodies of the invention can also be administered in the form of protein crystal formulations which include a combination of protein crystals encapsulated within a polymeric carrier to form coated particles. The coated particles of the protein crystal formulation may have a spherical morphology and be microspheres of up to 500 micro meters in diameter or they may have some other morphology and be microparticulates. The enhanced concentration of protein crystals allows the antibody of the invention to be delivered subcutaneously. In one embodiment, the TNFα antibodies of the invention are delivered via a protein delivery system, wherein one or more of a protein crystal formulation or composition, is administered to a subject with a TNFα-related disorder. Compositions and methods of preparing stabilized formulations of whole

antibody crystals or antibody fragment crystals are also described in WO 02/072636, which is incorporated by reference herein. In one embodiment, a formulation comprising the crystallized antibody fragments described in Examples 37 and 38 are used to treat a TNFα-related disorder.

In certain embodiments, an antibody, antibody portion, or other TNFα inhibitor of the invention may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The compound (and other ingredients, if desired) may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. To administer a compound of the invention by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation.

The pharmaceutical compositions of the invention may include a "therapeutically effective amount" or a "prophylactically effective amount" of an antibody or antibody portion of the invention. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the antibody, antibody portion, or other TNFα inhibitor may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the antibody, antibody portion, other TNFα inhibitor to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody, antibody portion, or other TNFα inhibitor are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

Dosage regimens may be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be

proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

An exemplary, non-limiting range for a therapeutically or prophylactically effective amount of an antibody or antibody portion of the invention is 10-150 mg, more preferably 20-80 mg and most preferably about 40 mg. It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition. Ranges intermediate to the above recited concentrations, *e.g.*, about 6-144 mg/ml, are also intended to be part of this invention. For example, ranges of values using a combination of any of the above recited values as upper and/or lower limits are intended to be included.

The invention also pertains to packaged pharmaceutical compositions which comprise a TNFα inhibitor of the invention and instructions for using the inhibitor to treat pulmonary disorders, including idiopathic interstitial lung diseases and chronic obstructive airway disorders, as described above.

Another aspect of the invention pertains to kits containing a pharmaceutical composition comprising an anti-TNFα antibody and a pharmaceutically acceptable carrier and one or more pharmaceutical compositions each comprising a drug useful for treating a pulmonary disorder and a pharmaceutically acceptable carrier. Alternatively,

the kit comprises a single pharmaceutical composition comprising an anti-TNFα antibody, one or more drugs useful for treating an inflammatory disorder and a pharmaceutically acceptable carrier. The kits contain instructions for dosing of the pharmaceutical compositions for the treatment of a pulmonary disorder in which the administration of an anti-TNFα antibody is beneficial, such as an idiopathic interstitial lung disease or a chronic obstructive airway disorder.

Another aspect of the invention pertains to kits containing a pharmaceutical composition comprising a TNFα antibody and a pharmaceutically acceptable carrier and one or more pharmaceutical compositions each comprising a drug useful for treating an inflammatory disorder and a pharmaceutically acceptable carrier. In one embodiment, the kit comprises a single pharmaceutical composition comprising an anti-TNFα antibody, one or more drugs useful for treating a pulmonary disorder, including idiopathic interstitial lung disease and chronic obstructive airway disorders, and a pharmaceutically acceptable carrier. The kits contain instructions for dosing of the pharmaceutical compositions for the treatment of a disorder in which the administration of an anti-TNFα antibody is beneficial, such as a pulmonary disorder, including idiopathic interstitial lung diseases and chronic obstructive airway disorders. The package or kit alternatively can contain the TNFα inhibitor and it can be promoted for use, either within the package or through accompanying information, for the uses or treatment of the disorders described herein. The packaged pharmaceuticals or kits further can include a second agent (as described herein) packaged with or copromoted with instructions for using the second agent with a first agent (as described herein).

B. *Additional therapeutic agents*

The invention pertains to pharmaceutical compositions and methods of use thereof for the treatment of a pulmonary disorder, including idiopathic interstitial lung diseases and chronic obstructive airway disorders. The pharmaceutical compositions comprise a first agent that prevents or inhibits a pulmonary disorder. The pharmaceutical composition also may comprise a second agent that is an active pharmaceutical ingredient; that is, the second agent is therapeutic and its function is beyond that of an inactive ingredient, such as a pharmaceutical carrier, preservative,

diluent, or buffer. The second agent may be useful in treating or preventing pulmonary disorders including idiopathic interstitial lung diseases and chronic obstructive airway disorders. The second agent may diminish or treat at least one symptom(s) associated with the targeted disease. The first and second agents may exert their biological effects by similar or unrelated mechanisms of action; or either one or both of the first and second agents may exert their biological effects by a multiplicity of mechanisms of action. A pharmaceutical composition may also comprise a third compound, or even more yet, wherein the third (and fourth, etc.) compound has the same characteristics of a second agent.

It should be understood that the pharmaceutical compositions described herein may have the first and second, third, or additional agents in the same pharmaceutically acceptable carrier or in a different pharmaceutically acceptable carrier for each described embodiment. It further should be understood that the first, second, third and additional agent may be administered simultaneously or sequentially within described embodiments. Alternatively, a first and second agent may be administered simultaneously, and a third or additional agent may be administered before or after the first two agents.

The combination of agents used within the methods and pharmaceutical compositions described herein may have a therapeutic additive or synergistic effect on the condition(s) or disease(s) targeted for treatment. The combination of agents used within the methods or pharmaceutical compositions described herein also may reduce a detrimental effect associated with at least one of the agents when administered alone or without the other agent(s) of the particular pharmaceutical composition. For example, the toxicity of side effects of one agent may be attenuated by another agent of the composition, thus allowing a higher dosage, improving patient compliance, and improving therapeutic outcome. The additive or synergistic effects, benefits, and advantages of the compositions apply to classes of therapeutic agents, either structural or functional classes, or to individual compounds themselves.

Supplementary active compounds can also be incorporated into the compositions. In certain embodiments, an antibody or antibody portion of the invention is coformulated with and/or coadministered with one or more additional therapeutic agents that are

useful for treating pulmonary disorders in which TNFα activity is detrimental, including idiopathic interstitial lung diseases and chronic obstructive airway disorders. For example, an anti-hTNFα antibody, antibody portion, or other TNFα inhibitor of the invention may be coformulated and/or coadministered with one or more additional antibodies that bind other targets (*e.g.*, antibodies that bind other cytokines or that bind cell surface molecules), one or more cytokines, soluble TNFα receptor (see *e.g.*, PCT Publication No. WO 94/06476) and/or one or more chemical agents that inhibit hTNFα production or activity (such as cyclohexane-ylidene derivatives as described in PCT Publication No. WO 93/19751). Furthermore, one or more antibodies or other TNFα inhibitors of the invention may be used in combination with two or more of the foregoing therapeutic agents. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible toxicities or complications associated with the various monotherapies. Specific therapeutic agent(s) are generally selected based on the particular pulmonary disorder being treated, as discussed below.

Nonlimiting examples of therapeutic agents with which an antibody, antibody portion, or other TNFα inhibitor of the invention can be combined include the following: non-steroidal anti-inflammatory drug(s) (NSAIDs); cytokine suppressive anti-inflammatory drug(s) (CSAIDs); CDP-571/BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2/infliximab (chimeric anti-TNFα antibody; Centocor); 75 kdTNFR-IgG/etanercept (75 kD TNF receptor-IgG fusion protein; Immunex; see *e.g.*, *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kdTNF-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); IDEC-CE9.1/SB 210396 (non-depleting primatized anti-CD4 antibody; IDEC/SmithKline; see *e.g.*, *Arthritis & Rheumatism* (1995) Vol. 38, S185); DAB 486-IL-2 and/or DAB 389-IL-2 (IL-2 fusion proteins; Seragen; see *e.g.*, *Arthritis & Rheumatism* (1993) Vol. 36, 1223); Anti-Tac (humanized anti-IL-2Rα; Protein Design Labs/Roche); IL-4 (anti-inflammatory cytokine; DNAX/Schering); IL-10 (SCH 52000; recombinant IL-10, anti-inflammatory cytokine; DNAX/Schering); IL-4; IL-10 and/or IL-4 agonists (*e.g.*, agonist antibodies); IL-1RA (IL-1 receptor antagonist; Synergen/Amgen); TNF-bp/s-TNFR (soluble TNF binding protein; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement),

S284; *Amer. J. Physiol. - Heart and Circulatory Physiology* (1995) Vol. 268, pp. 37-42); R973401 (phosphodiesterase Type IV inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); MK-966 (COX-2 Inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S81); Iloprost (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S82); methotrexate; thalidomide (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282) and thalidomide-related drugs (*e.g.*, Celgen); leflunomide (anti-inflammatory and cytokine inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S131; *Inflammation Research* (1996) Vol. 45, pp. 103-107); tranexamic acid (inhibitor of plasminogen activation; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S284); T-614 (cytokine inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); prostaglandin E1 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); Tenidap (non-steroidal anti-inflammatory drug; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S280); Naproxen (non-steroidal anti-inflammatory drug; see *e.g.*, *Neuro Report* (1996) Vol. 7, pp. 1209-1213); Meloxicam (non-steroidal anti-inflammatory drug); Ibuprofen (non-steroidal anti-inflammatory drug); Piroxicam (non-steroidal anti-inflammatory drug); Diclofenac (non-steroidal anti-inflammatory drug); Indomethacin (non-steroidal anti-inflammatory drug); Sulfasalazine (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S281); Azathioprine (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S281); ICE inhibitor (inhibitor of the enzyme interleukin-1β converting enzyme); zap-70 and/or lck inhibitor (inhibitor of the tyrosine kinase zap-70 or lck); VEGF inhibitor and/or VEGF-R inhibitor (inhibitos of vascular endothelial cell growth factor or vascular endothelial cell growth factor receptor; inhibitors of angiogenesis); corticosteroid anti-inflammatory drugs (*e.g.*, SB203580); TNF-convertase inhibitors; anti-IL-12 antibodies; anti-IL-18 antibodies; interleukin-11 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S296); interleukin-13 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S308); interleukin-17 inhibitors (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S120); gold; penicillamine; chloroquine; hydroxychloroquine; chlorambucil; cyclophosphamide; cyclosporine; total lymphoid irradiation; anti-thymocyte globulin;

anti-CD4 antibodies; CD5-toxins; orally-administered peptides and collagen; lobenzarit disodium; Cytokine Regulating Agents (CRAs) HP228 and HP466 (Houghten Pharmaceuticals, Inc.); ICAM-1 antisense phosphorothioate oligodeoxynucleotides (ISIS 2302; Isis Pharmaceuticals, Inc.); soluble complement receptor 1 (TP10; T Cell Sciences, Inc.); prednisone; orgotein; glycosaminoglycan polysulphate; minocycline; anti-IL2R antibodies; marine and botanical lipids (fish and plant seed fatty acids; see *e.g.*, DeLuca *et al.* (1995) *Rheum. Dis. Clin. North Am.* 21:759-777); auranofin; phenylbutazone; meclofenamic acid; flufenamic acid; intravenous immune globulin; zileuton; mycophenolic acid (RS-61443); tacrolimus (FK-506); sirolimus (rapamycin); amiprilose (therafectin); cladribine (2-chlorodeoxyadenosine); azaribine; methotrexate; antivirals; and immune modulating agents. Any of the above-mentioned agents can be administered in combination with the TNFα antibody of the invention to treat an inflammatory disease, including, for example, a idiopathic interstitial lung disease or chronic obstructive airway disorder. Examples of idiopathic interstitial lung diseases or chronic obstructive airway disorders include idiopathic pulmonary fibrosis, asthma and chronic obstructive pulmonary disease.

In one embodiment, the TNFα antibody of the invention is administered in combination with one of the following agents for the treatment of rheumatoid arthritis: methotrexate; prednisone; celecoxib; folic acid; hydroxychloroquine sulfate; rofecoxib; etanercept; infliximab; leflunomide; naproxen; valdecoxib; sulfasalazine; methylprednisolone; ibuprofen; meloxicam; methylprednisolone acetate; gold sodium thiomalate; aspirin; azathioprine; triamcinolone acetonide; propxyphene napsylate/apap; folate; nabumetone; diclofenac; piroxicam; etodolac; diclofenac sodium; oxaprozin; oxycodone hcl; hydrocodone bitartrate/apap; diclofenac sodium/misoprostol; fentanyl; anakinra, human recombinant; tramadol hcl; salsalate; sulindac; cyanocobalamin/fa/pyridoxine; acetaminophen; alendronate sodium; prednisolone; morphine sulfate; lidocaine hydrochloride; indomethacin; glucosamine sulfate/chondroitin; cyclosporine; amitriptyline hcl; sulfadiazine; oxycodone hcl/acetaminophen; olopatadine hcl; misoprostol; naproxen sodium; omeprazole; mycophenolate mofetil; cyclophosphamide; rituximab; IL-1 TRAP; MRA; CTLA4-IG; IL-18 BP; ABT-874; ABT-325 (anti-IL 18); anti-IL 15; BIRB-796; SCIO-469; VX-702;

AMG-548; VX-740; Roflumilast; IC-485; CDC-801; and mesopram. In another embodiment, the TNFα antibody of the invention is administered for the treatment of a pulmonary disorder in combination with one of the above mentioned agents for the treatment of rheumatoid arthritis.

In one embodiment, the TNFα antibody of the invention is administered in combination with one of the following agents for the treatment of an idiopathic interstitial lung disease or a chronic obstructive airway disorder in which TNFα activity is detrimental: anti-IL12 antibody (ABT 874); anti-IL18 antibody (ABT 325); small molecule inhibitor of LCK; small molecule inhibitor of COT; anti-IL1 antibody; small molecule inhibitor of MK2; anti-CD19 antibody; small molecule inhibitor of CXCR3; small molecule inhibitor of CCR5; small molecule inhibitor of CCR11 anti-E/L selectin antibody; small molecule inhibitor of P2X7; small molecule inhibitor of IRAK-4; small molecule agonist of glucocorticoid receptor; anti-C5a receptor antibody; small molecule inhibitor of C5a receptor; anti-CD32 antibody; and CD32 as a therapeutic protein.

In yet another embodiment, the TNFα antibody of the invention is administered in combination with an antibiotic or antiinfective agent. Antiinfective agents include those agents known in the art to treat viral, fungal, parasitic or bacterial infections. The term, "antibiotic," as used herein, refers to a chemical substance that inhibits the growth of, or kills, microorganisms. Encompassed by this term are antibiotic produced by a microorganism, as well as synthetic antibiotics (e.g., analogs) known in the art. Antibiotics include, but are not limited to, clarithromycin (Biaxin®), ciprofloxacin (Cipro®), and metronidazole (Flagyl®).

In another embodiment, the TNFα antibody of the invention is administered in combination with an additional therapeutic agent to treat asthma. Examples of agents which can be used to reduce or inhibit the symptoms of asthma include the following: albuterol; salmeterol/fluticasone; sodium; fluticasone propionate; budesonide; prednisone; salmeterol xinafoate; levalbuterol hcl; sulfate/ipratropium; prednisolone sodium phosphate; triamcinolone acetonide; beclomethasone dipropionate; ipratropium bromide; Azithromycin; pirbuterol acetate, prednisolone, theophylline anhydrous, methylprednisolone sod succ, clarithromycin, zafirlukast, formoterol fumarate, influenza virus vaccine, methylprednisolone, trihydrate, flunisolide, allergy injection,

cromolyn sodium, fexofenadine hydrochloride, flunisolide/menthol, amoxicillin/clavulanate, levofloxacin, inhaler assist device, guaifenesin, dexamethasone sod phosphate; moxifloxacin hcl; doxyxyxline hyclate; guaifenesin/dextromethorphan; pseuduephedrine/codeine/chlorpheniramine; gatifloxacin; cetirizine hydrochloride; mometasone furoate; pseudoephedrine; salmeterol xinafoate; benzonatate; cephalexin; pseedoephedrine/hydrocodone/chlorpheniramine; cetirizine hcl/pseudoephedrine; phenylephrine/cod/promethazine; codeine/promethazine; cefprozil; dexamethasone; guaifenesin/pseudoephedrine, chlorpheniramine/hydrocodone, nedocromil sodium, terbutaline sulfate, epinephrine and methylprednisolone, metaproterenol sulfate; intravenous or intramuscular immunoglobulins, montelukast sodium.

In another embodiment, the TNFα antibody of the invention is administered in combination with an additional therapeutic agent to treat COPD. Examples of agents which can be used to reduce or inhibit the symptoms of COPD include, albuterol sulfate/ipratropium; ipratropium bromide; salmeterol/fluticasone; albuterol; salmeterol; xinafoate; fluticasone propionate; prednisone; theophylline anhydrous; methylprednisolone sod succ; montelukast sodium; budesonide; formoterol fumarate; triamcinolone acetonide; levofloxacin; guaifenesin; azithromycin; beclomethasone; dipropionate; levalbuterol hcl; flunisolide; sodium; trihydrate; gatifloxacin; zafirlukast; amoxicillin/clavulanate; flunisolide/menthol; chlorpheniramine/hydrocodone; metaproterenol sulfate; methylprednisolone; furoate; -ephedrine/cod/chlorphenir; pirbuterol acetate; -ephedrine/loratadine; terbutaline sulfate; tiotropium bromide;(R,R)-formoterol; TgAAT; Cilomilast and Roflumilast

In another embodiment, the TNFα antibody of the invention is administered in combination with an additional therapeutic agent to treat IPF. Examples of agents which can be used to reduce or inhibit the symptoms of IPF include prednisone; azathioprine; albuterol; colchicines; sulfate; digoxin; gamma interferon; methylprednisolone sod succ; furosemide; lisinopril; nitroglycerin; spironolactone; cyclophosphamide; ipratropium bromide; actinomycin d; alteplase; fluticasone propionate; levofloxacin; metaproterenol sulfate; morphine sulfate; oxycodone hcl; potassium chloride; triamcinolone acetonide; tacrolimus anhydrous; calcium; interferon-alpha; methotrexate; mycophenolate mofetil.

TNFα is detrimental, including idiopathic interstitial lung diseases and chronic obstructive airway disorders, in combination with the TNFα antibody of the invention. In one embodiment, any one of the above-mentioned therapeutic agents, alone or in combination therewith, can be administered to a subject suffering from rheumatoid arthritis in addition to a TNFα antibody to treat an pulmonary disease, including a idiopathic interstitial lung disease or chronic obstructive airway disorder. In another embodiment, any one of the above-mentioned therapeutic agents, alone or in combination therewith, can be administered in combination with the TNFα antibody of the invention, to a subject suffering from a pulmonary disease, such as a idiopathic interstitial lung disease or chronic obstructive airway disorder.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are incorporated herein by reference

EXAMPLES

Example 4: TNFα Inhibitor in Mouse Model for Asthma

TNF antibody study using ovalbumin (OVA)-induced allergic asthma mice

The mouse OVA model of allergic asthma (Hessel, E.M., *et al.* (1995) *Eur. J. Pharmacol.* 293:401; Daphne, T., *et al.* (2001) *Am. J. Respir. Cell Mol. Biol.* 25:751, is used in the following study for treating allergic asthma.

All mice are sensitized to OVA (chicken egg albumin, crude grade V; Sigma, St. Louis, MO). Active sensitization is performed without an adjuvant by giving seven intraperitoneal injections of 10 μg OVA in 0.5 ml pyrogen-free saline on alternate days (one injection per day). Three weeks after the last sensitization, mice are exposed to either 16 OVA challenges (2 mg/ml in pyrogen-free saline) or 16 saline aerosol challenges for 5 min on consecutive days (one aerosol per day). An additional group of

mice first receive eight OVA aerosols, followed by eight saline aerosols (OVA/saline, spontaneous resolution group).

For the experiment in the more severe ongoing model of allergic asthma, all mice are sensitized to OVA by active sensitization with two intraperitoneal injections (7 d apart) of 0.1 ml alum-precipitated antigen, comprising 10 μg OVA adsorbed onto 2.25 mg alum (AlumImject; Pierce, Rockford, IL). Two weeks after the second sensitization, mice are exposed to either six OVA challenges (10 mg/ml in pyrogen-free saline) or six saline aerosol challenges for 20 min every third day (one aerosol every third day). An additional group of mice first receive three OVA aerosols, followed by three saline aerosols (OVA/saline, spontaneous resolution group).

The aerosol treatment is performed in a plexiglas exposure chamber (5 liter) coupled to a Pari LC Star nebulizer (PARI Respiratory Equipment, Richmond, VA; particle size 2.5-3.1 μm) driven by compressed air at a flow rate of 6 liters/min. Aerosol is given in groups composed of no more than eight animals.

A monoclonal anti-TNFα antibody which is known to bind and neutralize mouse TNFα, *e.g.*, antibody TN3 (TN3-19.12) (see Marzi *et* al. (1995) *Shock* 3:27; Williams *et al.* (1992) *Proc Natl Acad Sci U S A*. 89:9784; BD Biosciences Pharmingen) is administered to the OVA sensitized mice in a range of doses after the second sensitization according to standard protocols known in the art. Appropriate placebo controls are also administered.

Airway responsiveness is measured in conscious, unrestrained mice using barometric whole-body plethysmography by recording respiratory pressure curves in response to inhaled methacholine (acetyl-β-methylcholine chloride; Sigma). Briefly, mice are placed in a whole-body chamber, and basal readings are obtained and averaged for 3 min. Aerosolized saline, followed by doubling concentrations of methacholine (ranging from 1.6-50 mg/ml saline), are nebulized for 3 min, and readings are taken and averaged for 3 min after each nebulization. Dose-response curves (DRCs) to methacholine are statistically analyzed by a general linear model of repeated measurements followed by *post-hoc* comparison between groups. Data are LOG transformed before analysis to equalize variances in all groups.

After measurement of *in vivo* airway responsiveness, mice are sacrificed by intraperitoneal injection of 1 ml 10% urethane in pyrogen-free saline (Sigma). Subsequently, mice are bled by cardiac puncture, and OVA-specific IgE is measured by ELISA. Briefly, microtiter plates (Nunc A/S, Roskilde, Denmark) are coated overnight at 4°C with 2 μg/ml rat anti-mouse IgE (clone EM95) diluted in phosphate-buffered saline (PBS). The next day, the ELISA is performed at room temperature. After blocking with ELISA buffer (PBS containing 0.5% bovine serum albumin [Sigma], 2 mM EDTA, 136.9 mM NaCl, 50 mM Tris, 0.05% Tween-20 [Merck, Whitehouse Station, NJ] pH 7.2) for 1 h, serum samples and a duplicate standard curve (starting 1:10), diluted in ELISA buffer, are added for 2 h. An OVA-specific IgE reference standard is obtained by intraperitoneal immunization with OVA and arbitrarily assigned a value of 10,000 experimental units/ml (EU/ml). After incubation, 1 μg/ml of OVA coupled to digoxigenin (DIG), which is prepared from a kit containing DIG-3-*o*-methylcarbonyl-ε-aminocaproic acid-N-hydroxy-succinimide-ester (Roche Diagnostics, Basel, Switzerland) in ELISA buffer, is added for 1.5 h, followed by incubation with anti-DIG-Fab fragments coupled to horseradish peroxidase (Roche Diagnostics) diluted 1:500 in ELISA buffer for 1 hour. Color development is performed with *o*-phenylenediamine-dichloride substrate (0.4 mg/ml, Sigma) and 4 mM H_2O_2 in PBS and stopped by adding 4 M H_2SO_4. The optical density is read at 492 nm, using a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA). The detection limit of the ELISA is 0.5 EU/ml IgE.

Bronchial alveolar lavage (BAL) is performed immediately after bleeding of the mice. Briefly, the airways are lavaged five times through a tracheal cannula with 1-ml aliquots of pyrogen-free saline warmed to 37°C. The recovered lavage fluid is pooled, and cells are pelleted ($32 \times g$, 4°C, 5 min) and resuspended in 150 μl cold PBS. The total number of cells in the BALF is determined using a Bürker-Türk counting-chamber (Karl Hecht Assistent KG, Sondheim/Röhm, Germany). For differential BALF cell counts, cytospin preparations are made and stained with Diff-Quick (Dade AG, Düdingen, Switzerland). Per cytospin, 400 cells are counted and differentiated into mononuclear cells (monocytes, macrophages, and lymphocytes), eosinophils, and neutrophils by

standard morphology. Statistical analysis is performed using the nonparametric Mann-Whitney *U* test.

Cytokine production by antigen-restimulated T cells in lung tissue is determined as described previously (Hofstra, C.L., *et al.* (1999) *Inflamm. Res.* 48:602). Briefly, the lungs are lavaged as described above and perfused via the right ventricle with 4 ml saline containing 100 U/ml heparin to remove any blood and intravascular leukocytes. Complete lung tissue is removed and transferred to cold sterile PBS. Lungs are then minced and digested in 3 ml RPMI 1640 containing 2.4 mg/ml collagenase A and DNase I (grade II) (both from RocheDiagnostics) for 30 min at 37°C. Collagenase activity is stopped by adding fetal calf serum (FCS). The lung tissue digest is filtered through a 70-μm nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) with 10 ml RPMI 1640 to obtain a single-cell suspension. The lung-cell suspension is washed, resuspended in culture medium (RPMI 1640 containing 10% FCS, 1% glutamax I, and gentamicin [all from Life Technologies, Gaithersburg, MD]) and 50 mM β-mercaptoethanol (Sigma), and the total number of lung cells is determined using a Bürker-Türk counting-chamber. Lung cells (8×10^5 lung cells/well) are cultured in round-bottom 96-well plates (Greiner Bio-One GmbH, Kremsmuenster, Austria) in the presence of OVA (10 μg/ml) or medium only. As a positive control, cells are cultured in the presence of plate-bound rat anti-mouse CD3 (clone 17A2, 50 μg/ml, coated overnight at 4°C). Each *in vitro* stimulation is performed in triplicate. After 5 days of culture at 37°C, the supernatant is harvested, pooled per stimulation, and stored at −20°C until cytokine levels were determined by ELISA.

The IFN-γ, IL-4, IL-5, IL-10, and IL-13 ELISAs are performed according to the manufacturer's instructions (PharMingen, San Diego, CA). The detection limits of the ELISAs are 160 pg/ml for IFN-γ, 16 pg/ml for IL-4, 32 pg/ml for IL-5, and 100 pg/ml for IL-10 and IL-13.

In all experiments, airway responsiveness to methacholine, OVA-specific IgE levels in serum, cellular infiltration in the BALF, and T-cell responses in lung tissue are measured 24 hours after the last challenge in each mouse.

Improvements in asthma in the experimental mice are marked by a decrease in the number of mononuclear cells (including monocytes, macrophages, and lymphocytes),

eosinophils, and neutrophils in the BALF, a decrease in the airway hyperresponsiveness, and a decrease in the cytokine production by antigen-restimulated T cells in the lung tissue.

Example 5: TNFα Inhibitor in Mouse Model of Chronic Ostructive Pulmonary Disease (COPD)

Study examining treatment for alveolar enlargement and inflammation

The following study is performed using a cigarette smoke induced COPD mouse model (Keast, D.*et al.* (1981) *J. Pathol.* 135:249; Hautmaki, R.D., *et al.* (1997) *Science* 277:2002). In response to cigarette smoke, inflammatory cell recruitment into the lungs followed by pathologic changes characteristic of emphysema have been observed. Previous studies have shown that progressive inflammatory cell recruitment begins within the first month of smoking followed by air space enlargement after 3 to 4 months of cigarette exposure (Hautmaki *et al.* (1997) *Science* 277:2002).

Mice are exposed to smoke from two non-filtered cigarettes per day, 6 days per week, for 6 months, with the use of a smoking apparatus with the chamber adapted for mice. Nonsmoking, age-matched animals are used as controls. After 6 months of exposure to smoke as described above, a monoclonal anti-TNFα antibody which is known to bind and neutralize mouse TNFα, *e.g.*, antibody TN3 (TN3-19.12) (see Marzi *et* al. (1995) *Shock* 3:27; Williams *et al.* (1992) *Proc Natl Acad Sci U S A.* 89:9784; BD Biosciences Pharmingen) is administered in a range of doses according to standard protocols known in the art. An appropriate placebo control is also administered. Mice are administered the antibody treatment for a period of 21 days. Mice are sacrificed, followed by examination of lung volume and compliance, cytokine measurement, histological mucus index measurement, alveolar duct enlargement, air space measurement, alveolar and interstitial macrophage counts and alveolar size, as described below.

Following antibody treatment, bronchiolar lavage is performed on euthanized animals; the trachea is isolated by blunt dissection, and small caliber tubing is inserted

and secured in the airway. Two volumes of 1.0 ml of PBS with 0.1% BSA are instilled, gently aspirated, and pooled. Each BAL fluid sample is centrifuged, and the supernatants are stored in –70° until used. Cytokine measurements are as described in Example 5.

To determine lung volume and compliance, animals are anesthetized, the trachea is cannulated, and the lungs are ventilated with 100% O_2 via a "T" piece attachment. The trachea is then clamped and oxygen absorbed in the face of ongoing pulmonary perfusion. At the end of this degassing, the lungs and heart are removed en bloc and inflated with PBS at gradually increasing pressures from 0 to 30 cm. The size of the lung at each 5-cm interval is evaluated via volume displacement. An increase in the lung volume of treated animals compared to placebo treated control animals indicates an improvement in COPD.

For histological analysis, animals are sacrificed and a median sternotomy is performed, and right heart perfusion is accomplished with calcium- and magnesium-free PBS to clear the pulmonary intravascular space. The lungs are then fixed to pressure (25 cm) with neutral buffered 10% formalin, fixed overnight in 10% formalin, embedded in paraffin, sectioned at 5 μm and stained with Hematoxylin and eosin (H&E) and periodic acid-Schiff with diastase (D-PAS).

The histological mucus index (HMI) provides a measurement of the percentage of epithelial cells that are D-PAS$^+$ per unit airway basement membrane. It is calculated from D-PAS–stained sections (Cohn, L., *et al.* (1997) *J. Exp. Med.* 186:1737). A decrease in the HMI of treated animals compared to placebo treated control animals indicates an improvement in COPD.

Lm, an indicator of air space size, is calculated for each mouse from 15 random fields at ×200 by means of a 50-line counting grid (10-mm total length). The results are the average of measurements of two independent investigators. An increase in air space size of treated animals compared to placebo treated control animals indicates an improvement in COPD.

To determine alveolar duct enlargement, the proximal surface areas from the terminal bronchiole-alveolar duct transition extending 250 μm into the duct using Optimus 5.2 image analysis software (Optimus, Bothell, WA) is measured. A decrease

in alveolar duct size of treated animals compared to placebo treated control animals indicates an improvement in COPD.

Alveolar and interstitial macrophages are quantitated by counting macrophages identified by murine Mac-3 (rat antibody to mouse (0.5 mg/ml), used at 1:4000 dilution; PharMingen, San Diego, CA0 immunostaining using the avidin-biotin alkaline. A decrease in the number of alveolar and interstitial macrophages of treated animals compared to placebo treated control animals indicates an improvement in COPD.

Alveolar size is estimated from the mean cord length of the airspace (Ray, P., *et al.* (1997) *J. Clin. Invest.* 100:2501). This measurement is similar to the mean linear intercept, a standard measure of air space size, but has the advantage that it is independent of alveolar septal thickness. Sections are prepared as described above. To obtain images at random for analysis, each glass slide is placed on a printed rectangular grid and a series of dots placed on the coverslip at the intersection of the grid lines, *i.e.*, at intervals of approximately 1 mm. Fields as close as possible to each dot are acquired by systematically scanning at 2-mm intervals. Fields containing identifiable artifacts or non-alveolated structures such as bronchovascular bundles or pleura are discarded.

A minimum of ten fields from each mouse lung are acquired into a Macintosh G3 computer (Apple Computer Inc., Cupertino, California, USA) through a framegrabber board. Images are acquired in 8-bit gray-scale at a final magnification of 1.5 pixels per micron. The images are analyzed on a Macintosh computer using the public domain NIH Image program written by Wayne Rasband at NIH using a custom-written macro available from the web site (http://rsb.info.nih.gov/nih-image). Images are manually thresholded and then smoothed and inverted. The image is then subject to sequential logical image match "and" operations with a horizontal and then vertical grid. At least 300 measurements per field are made for each animal. The overlying air space air is averaged as the mean chord length. Chord length increases with alveolar enlargement. An increase in alveolar size of treated animals compared to placebo treated control animals indicates an improvement in COPD.

<u>Example 6: TNFα Inhibitor in Idiopathic Pulmonary Fibrosis (IPF) Mouse Model.</u>

Study of IPF treatment using bleomycin induced lung fibrosis mouse model

The following study is performed using the bleomycin induced lung fibrosis mouse model (reviewed in Bowden, D.H. (1984) *Lab. Invest.* 50:487; Tokuda, A., *et al.* (2000) *J. Immunol.* 164:2745).

Bleomycin sulfate is administered to C57BL/6J female mice aged 8–10 weeks. Briefly, C57BL/6J mice are anesthetized with 200 μl of 5 mg/ml pentobarbital injected i.p., followed by intratracheal instillation of 3 mg/kg bleomycin sulfate in 50 μl sterile saline.

A monoclonal anti-TNFα antibody which is known to bind and neutralize mouse TNFα, *e.g.,* antibody TN3 (TN3-19.12) (see Marzi *et* al. (1995) *Shock* 3:27; Williams *et al.* (1992) *Proc Natl Acad Sci U S A.* 89:9784; BD Biosciences Pharmingen) is administered to the bleomycin induced lung fibrosis mice in a range of doses, after intratracheal instillation of bleomycin as described above. An appropriate placebo control is also administered. Mice ar treated twice daily for 14 days.

Mice are sacrificed 20 and 60 days following bleomycin treatment. Tissues are fixed in 10% buffered formalin and embedded in paraffin. Sections are stained with hematoxylin and eosin and examined by light microscopy. Lung-infiltrating leukocyte counts, cytokine measurements, and total lung collagen content is determined as described below.

BAL cells and lung-infiltrating leukocytes are prepared as described in Smith *et al.* (1994) *J. Immunol.* 153:4704. In brief, following anesthesia, 1 ml PBS is instilled and withdrawn five times from the lung via an intratracheal cannula. The BAL fluids are collected and after RBC lysis total leukocyte counts are determined. Cell differentials are determined after cytospin centrifuge. Specimens are stained with Diff-Quik products (Baxter, Miami, FL).

To isolate lung-infiltrating leukocytes, lungs are perfused with saline, dissected from the chest cavity, and then minced with scissors. Each sample is incubated for 30 minutes at 37°C on a rocker in 15 ml digesting buffer (10% FCS in RPMI 1640 with 1% collagenase; Wako Pure Chemical, Osaka, Japan). Next, the sample is pressed through nylon mesh and suspended in 10% FCS-RPMI 1640 after being rinsed. The cell

suspension is treated with Histopaque-1119 (Sigma, St. Louis, MO) and centrifuged at 2000 rpm for 20 min to remove lung parenchymal cells and RBC. The pellet is resuspended in 2.5% FCS-PBS after being rinsed. After cell counts are performed, flow cytometric immunofluorescence analyses are conducted.

Immunofluorescence analyses of peripheral blood leukocytes and lung-infiltrating leukocytes are performed with the use of an Epics Elite cell sorter (Coulter Electronics, Hialeah, FL) as described previously (Yoneyama *et al.* (1998) *J. Clin. Invest.* 102:1933; Murai *et al.* (1999) *J. Clin. Invest.* 104:49). Peripheral blood leukocytes are prepared from normal mice with RBC lysis buffer. After incubation with Fc Block (anti-CD16/32; PharMingen, San Diego, CA) for 10 min, cells are stained with PE-conjugated mAb against CD3, CD4, CD8, CD11b, CD11c, and Gr-1 (PharMingen), and also stained with 20 μg/ml of rabbit anti-CCR1 polyclonal Ab followed by staining with FITC-conjugated goat anti-rabbit IgG (Leinco Technologies, St. Louis, MO). Before analyses propidium iodide (Sigma) staining is performed to remove the dead cells. A decrease in the number of lung-infiltrating leukocytes of treated animals compared to placebo treated control animals indicates an improvement in IPF.

Immunohistochemistry of lung samples is carried out as follows: lung specimens are prepared as described previously (Yoneyama *et al.* (1998) *J. Clin. Invest.* 102:1933; Murai *et al.* (1999) *J. Clin. Invest.* 104:49). Briefly, lung specimens are fixed in periodate-lysine-paraformaldehyde, washed with PBS containing sucrose, embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN), frozen in liquid nitrogen, and cut into 7-μm-thick sections with a cryostat. After inhibition of endogenous peroxidase activity, the sections are incubated with the first Ab. The Abs used are rabbit anti-CCR1 Ab, rat anti-F4/80 (BMA Biomedicals, Geneva, Switzerland), rat anti-CD4, rat anti-CD8, rat anti-Gr-1 (PharMingen), rat anti-nonlymphoid dendritic cell (NLDC)-145, and rat anti-MHC class II (BMA Biomedicals). As a negative control either a rabbit IgG or a rat IgG is used, respectively. They are treated sequentially with either HRP-conjugated goat anti-rabbit IgG (Cedarlane Laboratories, Hornby, Ontario, Canada) or a HRP-conjugated goat anti-rat IgG (BioSource International, Camarillo, CA). After staining with 3,3'-diaminobenzidine (Wako Pure Chemical) or 3-amino-9-ethylcarbazole substrate kit (Vector Laboratories, Burlingame, CA), samples are counterstained with Mayer's

hematoxylin. A decrease in CCR1, and decreases in the number of CD4+ T cells, , CD8+ T cells, nonlymphoid dendritic cell (NLDC), and MHC class II bearing cells of treated animals compared to placebo treated control animals indicates an improvement in IPF

Total lung collagen content is determined by assaying total soluble collagen using the Sircol Collagen Assay kit (Biocolor, Northern Ireland) according to the manufacturer's instructions. Briefly, lungs are harvested at day14 after bleomycin administration and homogenized in 10 ml 0.5 M acetic acid containing about 1 mg pepsin/10 mg tissue residue. Each sample is incubated for 24 h at 4°C with stirring. After centrifugation, 200 μl of each supernatant is assayed. One milliliter of Sircol dye reagent that binds to collagen is added to each sample and then mixed for 30 min. After centrifugation, the pellet is suspended in 1 ml of the alkali reagent included in the kit and read at 540 nm by a spectrophotometer. Collagen standard solutions are utilized to construct a standard curve. Collagens contain about 14% hydroxyproline by weight, and collagen contents obtained with this method correlate well with the hydroxyproline content according to the manufacturer's data. A decrease on lung collagen content of treated animals compared to placebo treated control animals indicates an improvement in IPF

Using the bleomycin induced lung fibrosis mouse model, mice are examined for a decrease in the BAL cell counts, a decrease in the peripheral blood leukocytes and lung infiltrating leukocytes. Mice are also examined for a decrease in the total lung collagen content in D2E7 treated mice as compared to placebo treated mice.

Example 7: TNFα Inhibitor in Treatment of Asthma

Clinical study of D2E7 in human subjects with asthma

Patients 12 to 65 years of age are eligible for the study if they have had a documented diagnosis of asthma of at least 2 years duration and have also had demonstrable reversible bronchospasm with an increase in FEV1 of 15% or greater after the administration of albuterol within the previous six months. Additional inclusion

criteria include, a baseline FEV1 between 50% and 80% of predicted normal, absence of any clinically significant disease other than asthma, a history of daily use of inhaled corticosteroids and cessation of all β2-agonist use 30 days prior to the beginning of the study.

A baseline visit occurs within 7 days after the screening visit. All patients undergo evaluation of FEV1 and have a complete physical examination. Pulmonary auscultation and oropharyngeal examinations are performed, and asthma symptoms are assesses. Patients who qualify are randomly assigned to a treatment group including a placebo group.

Following baseline measurements, patients begin receiving treatment. They are randomized and treated with either D2E7 or placebo in a blinded fashion. At days 15 and 29, all examinations performed at the baseline visit are repeated. A 12-lead ECG is also performed. Diary cards are reviewed with patients regarding the use of other medications and any adverse events.

Improvements are determined on spirometry tests measured at each visit. These include FEV1, peak expiratory flow rate (PEFR), Forced Vital Capacity (FCV), and forced expiratory flow at 25% to 75% of FVC. FEV1 at the final visit is regarded as the primary measure of efficacy. Twice-daily PEFR tests performed by the patient are compared and the number of inhalations of rescue medication is calculated. Patient/physician evaluations of asthma symptoms (wheezing, tightness in the chest, shortness of breath and cough) are characterized by severity. Compliance is assessed by review of the patient's diary cards and by collecting unused study medication.

<u>Example 8: TNFα Inhibitor in Treatment of COPD</u>

Clinical study examining D2E7 in human subjects with COPD

The study population is male and female subjects who are 40 to 80 years of age with a diagnosis of COPD. Subjects must have a best FEV1/FVC ratio ≤0.70 liters, fixed airway obstruction, defined by ≤15% or ≤200 ml (or both) increase in FEV1 after the administration of albuterol and a post-albuterol FEV1 between 30 and 70% of

predicted. Subjects must also be current or previous smokers with a history of smoking $\geq$10 pack years.

Following baseline measurements, patients begin receiving treatment. They are randomized and treated with either D2E7 or placebo in a blinded fashion.

Improvements are marked by an increase from predose baseline after study medication in pre-bronchodilator FEV1 and change from baseline in total score of the St. George's Respiratory Questionnaire (Jones, P.W., *et al.* (1991) *Resp. Med.* 85(suppl):25) which indicates an improvement in the patients' quality of life. Improvements are also seen as an increase from baseline FVC at trough, an increase in time to first COPD exacerbation, and a decrease from baseline in post-exercise breathlessness (modified Borg Scale; Stulbarg, M., Adams, L. Dyspnea. In: Murray J, Nadel J, eds. Textbook of Respiratory Medicine. Philadelphia, PA: WB Saunders, 2000; 541–552). Measures of safety are adverse events, vital signs, electrocardiogram at all double-blind visits, and laboratory assessments.

Example 9: TNFα Inhibitor in Treatment of IPF

Clinical study of D2E7 in human subjects with IPF.

A multi-center, double-blind, placebo-controlled study comparing treatment of IPF patients with D2E7 versus treatment with placebo is performed. Patients are eligible for the study if they have histologically verified IPF and have a decline in lung function of at least 10% during the 12 months prior to the beginning of the study, despite continuous or repeated treatment with glucocorticoids or other immunosuppressive agents or both for at least 6 months. The main histological feature used to identify IPF is the presence of subpleural and periacinar fibrotic lesions with only minor cellular infiltration. The absence of bilateral patchy infiltrates on high-resolution computed tomography and the demonstration of predominantly peripheral distribution of lesions are the radiological criteria for identifying the disease. Patients with a history of exposure to organic or inorganic dust or drugs known to cause pulmonary fibrosis and those with connective-tissue disease or other chronic lung diseases are excluded.

Patients with end-stage IPF as identified on the basis of a total lung capacity of less than 45% of the predicted normal are also excluded. Baseline values for repeat pulmonary function tests, FVC, total lung capacity (TLC), and oxygen saturation are taken.

Following baseline measurements, patients begin receiving treatment. They are randomized and treated with either D2E7 or placebo in a blinded fashion.

Improvements in IPF patients include an increase in the overall survival rate of patients in the study, and improvements in FVC, total lung capacity (TLC) and oxygen saturation. Improvement in pulmonary function is defined as a 10% or greater increase in predicted value of FVC or TLC, or a 3% or greater increase in oxygen saturation with the same fraction of expired air, resting or exertional. A decrease of similar manner for each measure is considered a deterioration. Patients who do not demonstrate improvement or deterioration are considered stable.

Example 7: Crystallization of D2E7 F(ab)'$_2$ fragment

Generation and purification of the D2E7 F(ab)'$_2$ Fragment

A D2E7 F(ab)'$_2$ fragment was generated and purified according to the following procedure. Two ml of D2E7 IgG (approximately 63 mg/ml) was dialyzed against 1 liter of Buffer A (20 mM NaOAc, pH 4) overnight. After dialysis, the protein was diluted to a concentration of 20 mg/ml. Immobilized pepsin (Pierce; 6.7 ml of slurry) was mixed with 27 ml of Buffer A, mixed, and centrifuged (Beckman floor centrifuge, 5000 rpm, 10 min). The supernatant was removed, and this washing procedure was repeated twice more. The washed immobilized pepsin was re-suspended in 13.3 ml of Buffer A. D2E7 (7.275 ml, 20 mg/ml, 145.5 mg) was mixed with 7.725 ml of Buffer A Bnd 7.5 ml of the washed immobilized pepsin slurry. The D2E7/pepsin mixture was incubated at 37 °C for 4.5 hr with shaking (300 rpm). The immobilized pepsin was then separated by centrifugation. Analysis of the supernatant by SDS-PAGE indicated that the digestion of D2E7 was essentially complete (~115 kDa band unreduced, ~30 and ~32 kDa bands reduced).

The D2E7 F(ab)'$_2$ fragment was separated from intact D2E7 and Fc fragments using Protein A chromatography. One-half of the above reaction supernatant (10 ml)

was diluted with 10 ml of Buffer B (20 mM Na phosphate, pH 7), filtered through a 0.45 μm Acrodisk filter, and loaded onto a 5 ml Protein A Sepharose column (Pharmacia Hi-Trap; previously washed with 50 ml of Buffer B). Fractions were collected. After the protein mixture was loaded, the column was washed with Buffer B until the absorbance at 280 nm re-established a baseline. Bound proteins were eluted with 5 ml of Buffer C (100 mM citric acid, pH 3); these fractions were neutralized by adding 0.2 ml of 2 M Tris•HCl, pH 8.9. Fractions were analyzed by SDS-PAGE; those that contained the D2E7 F(ab)'$_2$ fragment were pooled (~42 ml). Protein concentrations were determined by absorbance at 280 nm in 6 M guanidine•HCl, pH 7 (calculated extinction coefficients: D2E7, 1.39 (AU-ml)/mg; F(ab)'$_2$, 1.36 (AU-ml)/mg). The flow-though pool contained ~38.2 mg protein (concentration, 0.91 mg/ml), which represents a 79% yield of F(ab)'$_2$ (theoretical yield is ~2/3 of starting material, divided by two [only half purified], i.e. ~48.5 mg).

The D2E7 F(ab)'$_2$ fragment was further purified by size-exclusion chromatography. The pooled Protein A flow-through was concentrated from ~42 to ~20 ml, and a portion (5 ml, ~7.5 mg) was then chromatographed on a Superdex 200 column (26/60, Pharmacia) previously equilibrated (and eluted) with Buffer D (20 mM HEPES, pH 7, 150 mM NaCl, 0.1 mM EDTA). Two peaks were noted by absorbance at 280 nm: Peak 1, eluting at 172–200 ml, consisted of F(ab)'$_2$ (analysis by SDS-PAGE; ~115 kDa band unreduced, ~30 and ~32 kDa bands reduced); Peak 2, eluting at 236–248 ml, consisted of low molecular weight fragment(s) (~15 kDa, reduced or unreduced). Peak 1 was concentrated to 5.3 mg/ml for crystallization trials.

Crystallization of the D2E7 F(ab)'$_2$ Fragment

The D2E7 F(ab)'$_2$ fragment (5.3 mg/ml in 20 mM HEPES, pH 7, 150 mM NaCl, 0.1 mM EDTA) was crystallized using the sitting drop vapor diffusion method by mixing equal volumes of F(ab)'$_2$ and crystallization buffer (approx. 1 μl of each) and allowing the mixture to equilibrate against the crystallization Buffer Bt 4 or 18 °C. The crystallization buffers used consisted of the Hampton Research Crystal Screens I (solutions 1–48) and II (solutions 1–48), Emerald Biostructures Wizard Screens I and II

(each solutions 1–48), and the Jena Biosciences screens 1–10 (each solutions 1–24). Crystals were obtained under many different conditions, as summarized in Table 1.

Table 1. Summary of crystallization conditions for the D2E7 F(ab)'$_2$ fragment.

Screen	Solution	Temp °C	Condition	Result
Hampton 1	32	4	2.0 M $(NH_4)_2SO_4$	tiny needle clusters
Hampton 1	46	4	0.2 M $Ca(Oac)_2$, 0.1 M Na cacodylate pH 6.5, 18% PEG 8K	medium sized needle clusters
Hampton 1	48	4	0.1 M Tris HCl pH 8.5, 2.0 M $NH_4H_2PO_4$	micro needle clusters
Hampton 2	2	4	0.01 M hexadecyltrimethylammonium bromide, 0.5 M NaCl, 0.01 M $MgCl_2$	small shard crystals
Hampton 2	13	4	0.2 M $(NH_4)_2SO_4$, 0.1 M NaOAc pH 4.6, 30% PEG MME 2000	small needle clusters
Hampton 2	15	4	0.5 M $(NH_4)_2SO_4$, 0.1M NaOAc pH 5.6, 1.0M Li_2SO_4	large needle clusters
Hampton 2	16	4	0.5M NaCl, 0.1M NaOAc pH 5.6, 4% Ethylene Imine polymer	large irregular crystal
Hampton 1	34	18	0.1 NaOAc pH 4.6, 2.0 M Na Formate	needle clusters
Hampton 1	35	18	0.1M Hepes pH 7.5, 0.8M mono-sodium dihydrogen phosphate, 0.8M mono-potasium dihydrogen phosphate	needle clusters
Hampton 2	9	18	0.1M NaOAc pH 4.6, 2.0M NaCl	dense needle clusters
Hampton 2	12	18	0.1M $CdCl_2$, 0.1M NaOAc pH 4.6, 30% PEG 400	needles & amorphous crystals
Hampton 2	15	18	0.5M $(NH_4)_2SO_4$, 0.1M NaOAc pH 5.6, 1.0M Li_2SO_4	tiny needle clusters
Wizard I	27	4	1.2M NaH2PO4, 0.8M K2HPO4, 0.1M CAPS pH 10.5, 0.2 M Li_2SO_4	Medium large needle clusters
Wizard I	30	4	1.26M $(NH_4)_2SO_4$, 0.1 M NaOAc pH 4.5, 0.2M NaCl	small needle clusters
Wizard II	8	4	10% PEG 8K, 0.1M Na/K phosphate pH 6.2, 0.2M NaCl	Large plate crystals grown in clusters
Wizard II	43	4	10% PEK 8K, 0.1M Tris pH 7.0, 0.2 M MgCl2	micro needle clusters
Wizard I	4	18	35% MPD, 0.1M Imidazole pH 8.0, 0.2M MgCl2	rod shaped crystal
Wizard I	27	18	1.2M NaH2PO4, 0.8M K2HPO4, 0.1M CAPS pH 10.5, 0.2 M Li_2SO_4	Needle clusters
Wizard II	7	18	30% PEG 3K, 0.1M Tris pH 8.5, 0.2M NaCl	tiny needle clusters
Wizard II	11	18	10% 2-propanol, 0.1M cacodylate pH 6.5, 0.2M Zn(Oac)2	tiny hexagonal or rhombohedral crystals
Wizard II	46	18	1.0M AP, 0.1M Imidazole pH 8.0, 0.2M NaCl	1 irregular crystal
JB 1	D6	4	30% PEG 3K, 0.1M Tris HCl pH 8.5, 0.2M Li_2SO_4	tiny needles in precipitate
JB 2	B6	4	20% PEG 4K, 0.1M Tris HCl pH 8.5, 0.2M Na Cacodylate	tiny needle cluster balls
JB 3	A1	4	8% PEG 4K, 0.8M LiCl, 0.1M Tris HCl pH 8.5	Large frost-like crystals
JB 3	B1	4	15% PEG 4K, 0.2M $(NH_4)_2SO_4$	tiny needle clusters
JB 3	D5	4	30% PEG 4K, 0.1M Na Citrate pH 5.6, 0.2M NH_4OAc	tiny needles in precipitate.
JB 4	B1	4	15% PEG 6K, 0.05M KCl, 0.01M $MgCl_2$	needle cluster balls
JB 3	A6	18	12% PEG 4K, 0.1M NaOAc pH 4.6, 0.2M NH_4OAc	needle clusters

Screen	Solution	Temp °C	Condition	Result
JB 3	B1	18	15% PEG 4K, 0.2M $(NH_4)_2SO_4$	needle clusters in precipitate
JB 3	C6	18	25% PEG 4K, 0.1M Na Citrate pH 5.6, 0.2M NH_4OAc	long, thin needles
JB 4	C5	18	8% PEG 8K, 0.2 M LiCl, 0.05M $MgSO_4$	frost-like crystals
JB 5	A3	4	15% PEG 8K, 0.2M $(NH_4)_2SO_4$	long single needles in phase separation
JB 5	A4	4	15% PEG 8K, 0.5M Li_2SO_4	tiny needle clusters
JB 5	A5	4	15% PEG 8K, 0.1M Na MES pH 6.5, 0.2M $Ca(OAc)_2$	needle cluster balls
JB 6	B2	4	1.6M $(NH_4)_2SO_4$, 0.5 LiCl	tiny needle cluster balls
JB 6	C2	4	2.0 M $(NH_4)_2SO_4$, 0.1M NaOAc pH 4.6	micro needle clusters
JB 10	D3	18	2.0M Na Formate, 0.1M NaOAc pH 4.6	needle clusters

The following conditions (as described in Table 1) produced crystals which can be used for diffraction quality crystals: Wizard II, 11, 18, 10% 2-propanol, 0.1M cacodylate pH 6.5, 0.2M Zn(Oac)2, tiny hexagonal or rhom. Xtals; Wizard II, 10% PEG 8K, 0.1M Na/K phosphate pH 6.2, 0.2M NaCl, large plate xtals grown in clusters; JB 3, C6, 18, 25% PEG 4K, 0.1M Na Citrate pH 5.6, 0.2M Ammonium Acetate, long, thin needles; Hampton 2, 15, 18, 0.5M AS, 0.1M Na Acetate trihydrate pH 5.6, 1.0M Li Sulfate monohydrate, tiny needle clusters.

Example 38: Crystallization of D2E7 Fab fragment

Generation and purification of the D2E7 Fab Fragment

A D2E7 Fab fragment was generated and purified according to the following procedure. Four ml of D2E7 IgG (diluted to about 20 mg/ml) was diluted with 4 ml of Buffer E (20 mM Na phosphate, 5 mM cysteine•HCl, 10 mM EDTA, pH7) and mixed with 6.5 ml of a slurry of immobilized papain (Pierce, 1%; previously washed twice with 26 ml of Buffer E). The D2E7/papain mixture was incubated at 37 °C overnight with shaking (300 rpm). The immobilized papain and precipitated protein were separated by centrifugation; analysis of the supernatant by SDS-PAGE indicated that the digestion of D2E7 was partially complete (~55, 50, 34, and 30 kDa bands unreduced, with some intact and partially digested D2E7 at ~115 and ~150 kDa; ~30 and ~32 kDa bands

reduced, as well as a ~50 kDa band). Nonetheless, the digestion was halted and subjected to purification.

The D2E7 Fab fragment was purified by Protein A chromatography and Superdex 200 size-exclusion chromatography essentially as described above for the F(ab)'$_2$ fragment. The Protein A column flow-through pool (21 ml) contained ~9.2 mg (0.44 mg/ml), whereas the Protein A eluate (4 ml) contained ~19.5 mg (4.9 mg/ml). Analysis by SDS-PAGE indicated that the flow-through was essentially pure Fab fragment (~48 and ~30 kDa unreduced, broad band at ~30 kDa reduced), whereas the eluate was intact and partially-digested D2E7. The Fab fragment was further purified on a Superdex 200 column, eluting at 216–232 ml, i.e., as expected, after the F(ab)'$_2$ fragment but before the small Fc fragments. The D2E7 Fab fragment concentrated to 12.7 mg/ml for crystallization trials, as described below.

Crystallization of the D2E7 Fab Fragment

The D2E7 Fab fragment (12.7 mg/ml in 20 mM HEPES, pH 7, 150 mM NaCl, 0.1 mM EDTA) was crystallized using the sitting drop vapor diffusion method essentially as described above for the F(ab)'$_2$ fragment. Crystals were obtained under many different conditions, as summarized in Table 2.

Table 2. Summary of crystallization conditions for the D2E7 Fab fragment.

Screen	Solution	Temp °C	Condition	Result
Hampton 1	4	4	0.1M Tris pH 8.5, 2M $(NH_4)_2SO_4$	wispy needles
Hampton 1	10	4	0.2M NH_4OAc, 0.1M NaOAc pH 4.6, 30% PEG 4K	wispy needle clusters
Hampton 1	18	4	0.2M $Mg(OAc)_2$, 0.1M Na Cacodylate pH 6.5, 20% PEG 8K	needle clusters
Hampton 1	20	4	0.2M $(NH_4)_2SO_4$, 0.1M NaOAc pH 4.6, 25% PEG 4K	tiny needle clusters
Hampton 1	32	4	2M $(NH_4)_2SO_4$	long, wispy needles
Hampton 1	33	4	4M Na Formate	tiny needle clusters
Hampton 1	38	4	0.1M Hepes pH 7.5	tiny needle clusters
Hampton 1	43	4	30% PEG 1500	tiny needle clusters
Hampton 1	46	4	0.2M $Ca(OAc)_2$, 0.1M Na Cacodylate pH 6.5, 18% PEG 8K	large plate clusters
Hampton 1	47	4	0.1M NaOAc pH 4.6, 2M $(NH_4)_2SO_4$	long, wispy needles
Hampton 2	1	4	2M NaCl, 10% PEG 6K	small plate clusters

Screen	Solution	Temp °C	Condition	Result
Hampton 2	2	4	0.01M Hexadecyltrimethylammonium bromide, 0.5M NaCl, 0.01 $MgCl_2$	round & irregular plates
Hampton 2	5	4	2M $(NH_4)_2SO_4$, 5% isopropanol	long fiber ropes
Hampton 2	13	4	0.2M $(NH_4)_2SO_4$, 0.1M NaOAc pH 4.6, 25% PEG MME 2K	tiny, wispy needle clusters
Hampton 2	14	4	0.2M K/Na Tatrate, 0.1M Na Citrate pH 5.6, 2M $(NH_4)_2SO_4$	tiny needle clusters
Hampton 2	27	4	0.01M $ZnSO_4$, 0.1 MES pH 6.5, 25% PEG MME 550	tiny needle clusters
Hampton 2	28	4	30% MPD	tiny needle clusters
Hampton 1	4	18	0.1M Tris pH 8.5, 2M $(NH_4)_2SO_4$	needle clusters
Hampton 1	9	18	0.2M NH_4OAc, 0.1M Na Citrate pH 5.6, 30% PEG 4K	needle clusters
Hampton 1	17	18	0.2M Li_2SO_4, 0.1M Tris pH 8.5, 30% PEG 4K	long, wispy needles
Hampton 1	32	18	2M $(NH_4)_2SO_4$	needle clusters
Hampton 1	33	18	4M Na Formate	tiny needle clusters
Hampton 1	38	18	0.1M Hepes pH 7.5	fiber bundles
Hampton 1	43	18	30% PEG 1500	tiny needle clusters
Hampton 1	47	18	0.1M NaOAc pH 4.6, 2M $(NH_4)_2SO_4$	tiny needle clusters
Hampton 2	1	18	2M NaCl, 10% PEG 6K	long, wispy needle clusters
Hampton 2	5	18	2M $(NH_4)_2SO_4$, 5% 2-propanol	tiny needle clusters
Hampton 2	9	18	0.1M NaOAc pH 4.6, 2M NaCl	long, wispy needles
Hampton 2	13	18	0.2M $(NH_4)_2SO_4$, 0.1M NaOAc pH 4.6, 25% PEG MME 2K	tiny needle clusters
Hampton 2	14	18	0.2M K/Na Tartrate, 0.1M Na Citrate pH 5.6, 2M $(NH_4)_2SO_4$	long wispy needles
Hampton 2	27	18	0.01M $ZnSO_4$, 0.1 MES pH 6.5, 25% PEG MME 550	tiny needle clusters
Wizard I	20	4	0.4M NaH_2PO_4/1.6M K_2HPO_4, 0.1M Imidazole pH 8, 0.2M NaCl	tiny needle clusters
Wizard I	28	4	20% PEG 3K, 0.1M Hepes pH 7.5, 0.2M NaCl	large orthorhombic plate clusters
Wizard I	31	4	20% PEG 8K, 0.1M phosphate citrate pH 4.2, 0.2M NaCl	wispy needle clusters
Wizard I	39	4	20% PEG 1K, 0.1M phosphate citrate pH 4.2, 0.2M Li_2SO_4	needle clusters
Wizard II	3	4	20% PEG 8K, 0.1M Tris pH 8.5, 0.2M $MgCl_2$	large hexagonal or orthorhombic plate cluster in phase sep
Wizard II	4	4	2M $(NH_4)_2SO_4$, 0.1M Cacodylate pH 6.5, 0.2 NaCl	tiny needle clusters
Wizard II	9	4	2M $(NH_4)_2SO_4$, 0.1M phosphate citrate pH 4.2	tiny, wispy needle clusters
Wizard II	28	4	20% PEG 8K, 0.1M MES pH 6, 0.2M $Ca(OAc)_2$	tiny needle clusters; large wispy needle clusters
Wizard II	35	4	0.8M NaH_2PO_4/1.2M K_2HPO_4, 0.1M NaOAc pH 4.5	tiny fiber bundles
Wizard II	38	4	2.5M NaCl, 0.1M NaOAc pH 4.5, 0.2M Li_2SO_4	long wispy needles
Wizard II	47	4	2.5M NaCl, 0.1M Imidazole pH 8, 0.2M $Zn(OAc)_2$	tiny needle clusters
Wizard I	6	18	20% PEG 3K, 0.1M Citrate pH 5.5	needle clusters
Wizard I	20	18	0.4M NaH_2PO_4/1.6M K_2HPO_4, 0.1M Imidazole pH 8, 0.2M NaCl	tiny needle clusters

Screen	Solution	Temp °C	Condition	Result
Wizard I	27	18	1.2M NaH_2PO_4/0.8M K_2HPO_4, 0.1M CAPS pH 10, 0.2M Li_2SO_4	wispy needle clusters
Wizard I	30	18	1.26M $(NH_4)_2SO_4$, 0.1M NaOAc pH 4.5, 0.2M NaCl	wispy needles
Wizard I	31	18	20% PEG 8K, 0.1M phosphate citrate pH 4.2, 0.2M NaCl	tiny needle clusters
Wizard I	33	18	2M $(NH_4)_2SO_4$, 0.1M CAPS pH 10.5, 0.2M Li_2SO_4	fiber bundles
Wizard I	39	18	20% PEG 1K, 0.1M phosphate citrate pH 4.2, 0.2M Li_2SO_4	needle clusters
Wizard II	4	18	2M $(NH_4)_2SO_4$, 0.1M Cacodylate pH 6.5, 0.2 NaCl	needle clusters
Wizard II	9	18	2M $(NH_4)_2SO_4$, 0.1M phosphate citrate pH 4.2	wispy needles
Wizard II	35	18	0.8M NaH_2PO_4/1.2M K_2HPO_4, 0.1M NaOAc pH 4.5	tiny needle clusters
Wizard II	38	18	2.5M NaCl, 0.1M NaOAc pH 4.5, 0.2M Li_2SO_4	tiny needle clusters

The following conditions (as described in Table 2) produced crystals which can be used for diffraction quality crystals: Hampton 2, 1, 4C, 2M NaCl, 10% PEG 6K, small plate clusters; Hampton 1 46, 4C, 0.2M Ca Acetate, 0.1M Na Cacodylate, pH 6.5, 18% PEG 8K, large plate clusters; Wizard I, 28, 4C, 20% PEG 3K, 0.1M Hepes pH 7.5, 0.2M NaCl, large orthorhombic plate clusters; Wizard II 3, 4C, 20% PEG 8K, 0.1M Tris pH 8.5, 0.2M $MgCl_2$, lrg hex or orth plate cluster in phase sep.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.